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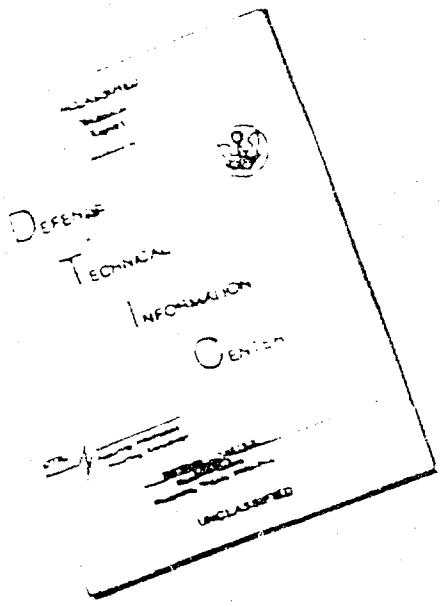

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13. ABSTRACT

The aim of the investigation are some of the factors involved in the conversion of mercury pollutants to toxic methyl-mercury by micro-organisms in marine sediments.

Research resulted in the definition of the two primary problems: modeling and analysis. - A successful modeling technique was then developed, after restrictions in design were found desirable. A successful analytical technique, which had not been applied as such to biological samples, was developed and used with good results. - These two solutions were then combined in an investigation of the conversion factors: 1. Pollutant type and level. 2 Conversion time. 3 Movement of water over the sediment (removal of Me-Hg produced). 4 Chelation. 5 Eutrophication. 6 Oxygen content of the water above the sediment.

The investigation has shown that long-term mercury pollution results primarily from solw conversion of large Hg^{+2} deposits to toxic methyl-mercury by methanogenic micro-organisms found largely in marine sediments. - The investigation into the biochemical reactions has shown that many pathways of conversion are possible under varying environmental conditions.

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"INVESTIGATION OF SOME FACTORS IN THE BIOCHEMICAL CONVERSION OF MERCURY POLLUTANTS TO TOXIC METHYL-MERCURY EFFECTED BY MICRO-ORGANISMS IN A MARINE SEDIMENT"

A Trident Scholar Project Report

by

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U. S. Naval Academy
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In thanking the Preparatory Room men, I appreciate their patience in handling some of my difficult requests for apparatus and reagents.

Finally, I appreciate the aid and criticism from Mrs. Linda Gedeon and Mrs. Sheryl Murray, secretaries in the Chemistry Department concerning the less technical and the more secretarial side of the project, especially in the frantic race to get the paper in on time.

M. McKinney

ABSTRACT

After comprehensive literature research and consultation with experts, project problems and goals were defined. The two basic problems were (1) setup of models to permit monitoring the production of methylmercury by sediment microorganisms and (2) development of a simple and rapid analytical method for mercury selective for alkyl-mercury compounds. To carry out the goal of investigating factors of conversion the solutions to the two basic problems were combined.

The basic problem of monitoring methylmercury production was solved after modeling experiments and design modifications. The solution was direct analysis for methylmercury of Rocky Gorge sediment in simple aquariums polluted with inorganic mercury, in which the factors of (1) concentration of pollutant, (2) time of conversion, (3) removal of loosely bound methylmercury by water motion, (4) presence of chelating agents, (5) eutrophication, and (6) oxygen content could be easily controlled.

The basic problem of development of the analytical method was solved by experimentation with the flameless atomic absorption method (AA method) and the gas chromatographic method (GC method) resulting in a combination of the two, giving sensitivity and specificity of 50 ng methyl-mercury/g sediment.

Combination of solutions resulted in partial attainment of the goal in that factors (1)-(3) above were satisfactorily investigated. Analytical results from (4)-(6) were obscured by interferences.

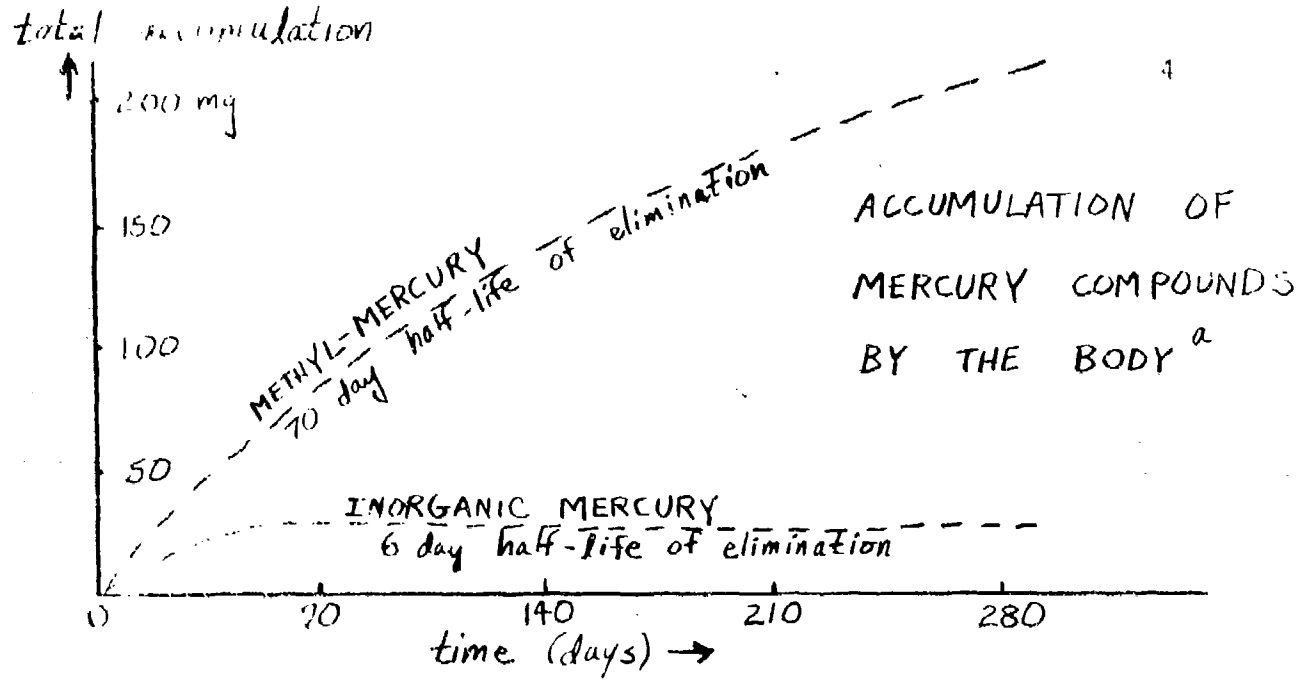
THE INVESTIGATION OF SOME FACTORS IN THE BIOCHEMICAL CONVERSION OF MERCURY POLLUTANTS TO TOXIC METHYL-MERCURY EFFECTED BY MICRO-ORGANISMS IN A MARINE SEDIMENT

Table of Contents

I. MERCURY IN THE ENVIRONMENT

A. HISTORICAL

Mercury (Hg), a heavy metal that is a liquid at room temperature (quicksilver), has played a significant role in man's technological advancement. It is very rare, composing (3) 10^{-8} of the earth's crust[1], and its properties have fascinated alchemists and chemists since medieval times. The toxicity of mercury and its compounds is well known; it has in times past been used for suicide and murder[2]. Elemental mercury (Hg°) is relatively harmless, while the most toxic compound of it known is methylmercury (CH_3Hg^+, Me-Hg). By comparison[3], a human being could ingest several hundred grams of Hg° without noticeable effects, yet the characteristic symptoms of organomercurial poisoning have appeared in blood levels as low as (2) 10^{-7} g/gram, corresponding to a daily intake of (3) 10^{-5} g of Me-Hg, for a normal man[4]. Mercury in the form of Hg^{++} (aqueous solution) is much more toxic than Hg°, but is not nearly of the order of toxicity of the Me-Hg form, which is well-documented but far from being completely understood. Organic mercury binds strongly to substances within the body; Me-Hg has a special affinity for the thiol group (-SH), found especially in proteins. This means that, while the half-life of elimination of Hg^{+2} by the body is 6 days, Me-Hg in the liver has a half-life of 70 days and in the brain a half-life of about 150 days[5].



This means that symptoms of severe organic-Hg poisoning (3 · 20 μg/g of brain tissue) will appear before 3 months at a (2) 10^{-1}g daily ingestion rate, while Hg^{+2} is eliminated quickly enough so that the body would never acquire more than (2) 10^{-1}g total, no symptoms appearing[6]. The redundant nature of the nervous system (where Me-Hg primarily has its effects) allows for some damage by poisoning to go unnoticed until later in life.

Prick, Sonner, and Slooff (1966) have made survey describing and listing case histories[7]. Most are laboratory workers, who had accidentally contacted Me-Hg or inhaled dimethyl-Hg (Me-Hg-Me, b.p. 96°C), or agricultural/industrial workers, who were poisoned by chronic and careless contact with such compounds, when they were used as slimicides/fungicides in seed treatment and other prevention of micro-biolog... growth. The classes of mercury and its compounds and some of their uses are[8]:

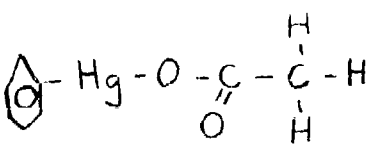
(1) Inorganic compounds (Hg°, HgX_2). Use in laboratory, instrument industry, medicine, catalysts, paints, electrical equipment. Most used as a catalyst for chlorine-caustic soda manufacture. Large losses despite recycle.

(2) Organic compounds. Uses are primarily dissipative.

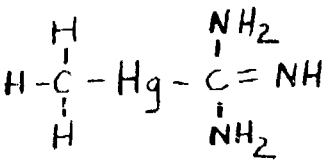
a. Alkyl-Hg-X. Methyl-mercury-dicyanodiamide (Panogen), a seed dressing used until the late 1960's.

b. Aryl-Hg-X. Phenyl-mercury-acetate, fungicide in paper industry used until late 1960's.

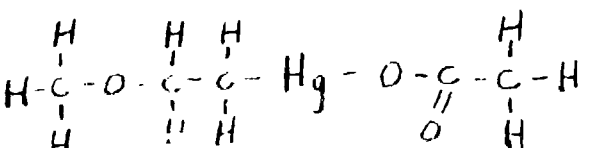
c. Alkoxyalkyl-Hg-X. Methyl-mercury-acetate, replaced other mercury compounds for seed dressing.



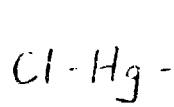
Phenyl-mercuric-acetate



Methyl-mercury-dicyanodiamide

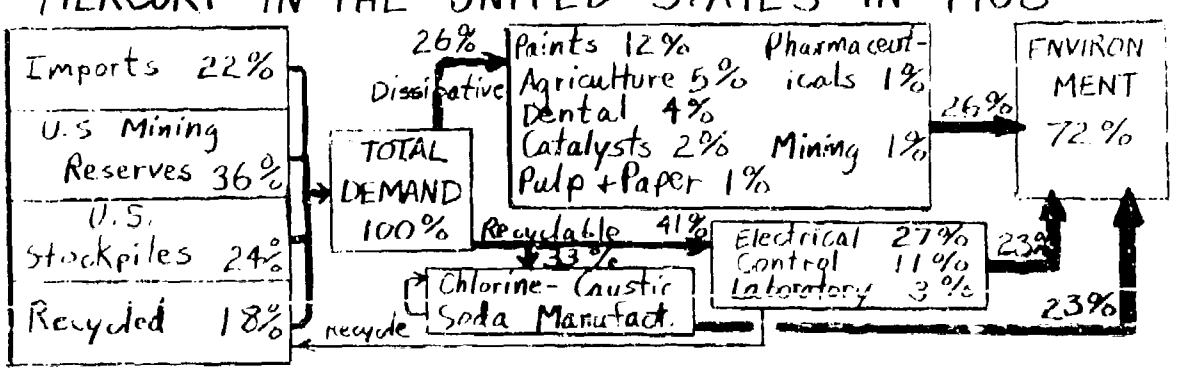


Methoxyethyl-mercuric-acetate

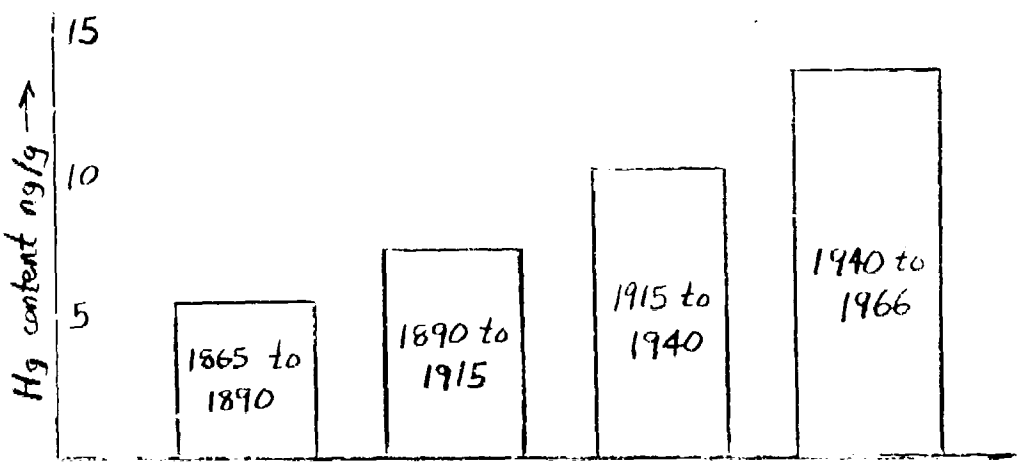


Mercuric Chloride

MERCURY IN THE UNITED STATES IN 1968[b]



From the chart it can be seen that most mercury released to the environment by humans is inorganic (Hg^{+2}), mostly due to ineffectiveness of recycling processes. About $(1.63)\ 10^8$ pounds of mercury have been used by the United States alone since 1900. About 10^6 pounds is annually added by the burning of fossil fuels. Though this is not enough to affect natural levels throughout the world, local marine environments may experience many, many times the natural level. The most common disposal/loss is via the water effluent. It was previously supposed that mercury, because of its physical and chemical properties, settled into inert pools on the sediment in the marine environment. When wild bird populations were noted to be declining in Sweden in the middle 1950's, Westoo and other investigators traced it to the use of mercury compounds as pesticides[9,10,11,12,13,14,15,16] They showed a decrease in mercury findings after restriction of alkyl-Hg in the use of seed dressing began in 1965.

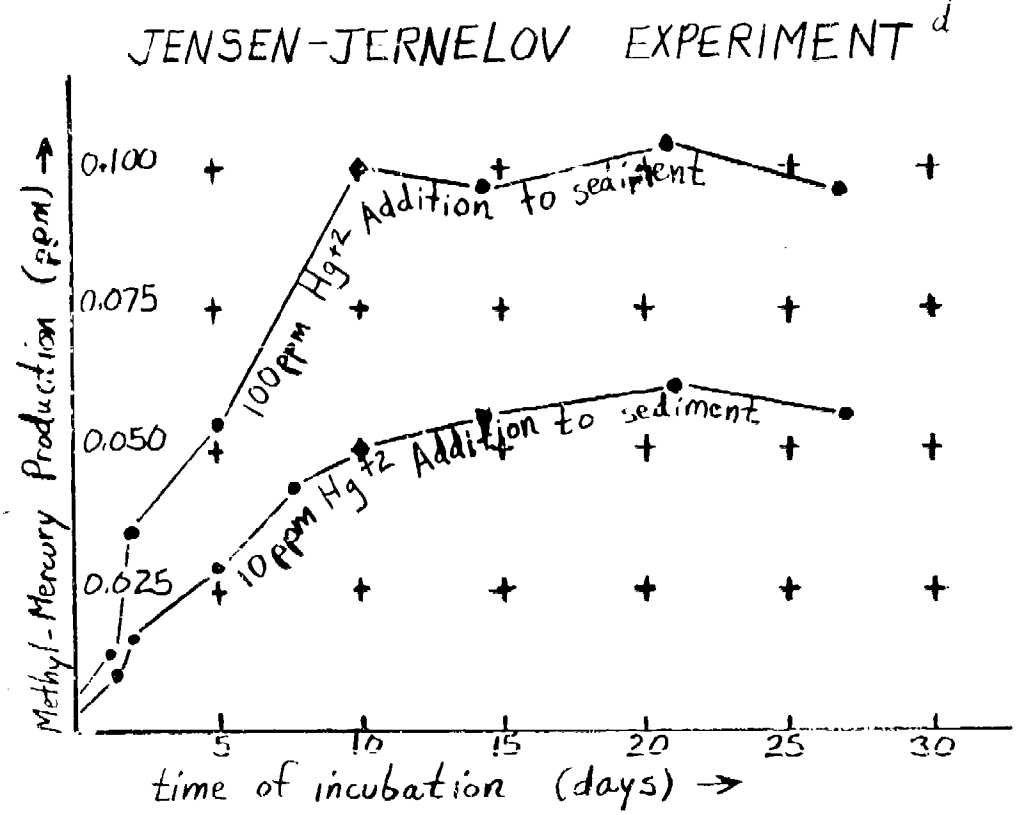


MERCURY IN FEATHERS OF GREAT CRESTED GREBES[C]

But mercury was being found in fish, also. At Minamata Bay, Japan, 121 people were killed or severely poisoned due to methyl-mercury being dumped as waste from a plastics plant directly into the bay. Marine animals regularly caught and eaten by the bay residents had concentrated the Me-Hg to toxic levels. The possibility of natural methylation of Hg was discarded here because of the source of pollution[12,18,19,20,21]. A similar incident occurrenced in 1970 along the Agano River in the Niigata prefecture with 47 people affected[22], and was interpreted the same way. Other such incidents occurred in Iraq, Pakistan, and Guatemala and were traced directly to Me-Hg[23,24,25], hence its abandonment in agricultural and industrial use. Sweden, having discovered its problem and begun research sooner than other countries, held a conference in Stockholm in 1965. Mercury was reported in many varieties of food, including fish, beef, and other meats. Westoo had recently shown that most of the mercury (80-90+%) in fish and eggs to be Me-Hg[26,27,28]. Mercury from marine products in other parts of the nonpolluted world were found to be mainly Me-Hg (in Japan[29], in the Indian Ocean[30]). Westoo held that fish and mammals could convert Hg^{+2} to Me-Hg in their livers[31]. Johnels and Olsson thus formed an opinion that such conversion could also be effected by micro-organisms in an anaerobic environment, e.g. a marine sediment[32]. This would make the fact that Hg^{+2} was the primary pollutant (mostly from chlorine-caustic soda manufacture) become very significant. This same year Sweden banned alkyl-Hg-X from agriculture[33].

U.S. Delegates attended the Swedish International Symposium (1966), but the Swedish concern did not spread to them, despite the fact that the United States primary use for mercury was for chlorine-caustic soda manufacture (15.1%)[34]. The FDA had already cut down on slimicides but agricultural use far exceeded that of the Swedes (400 tons/(3) 10^6 acres vs. 2 tons/(7.5) 10^6acres)[35].

In 1967 Jensen and Jernelov (Sweden) experimentally confirmed the Johnels-Olsson theory. Rosen (Sweden) communicated this to Wood[36] (biochemist at the University of Illinois) and with a NSF grant, Wood, Rosen, and Kennedy showed again (1968) that methanogenic bacteria could methylate Hg° and Hg^{+2}.



The importance of this remained overlooked in the U.S. until 1969. The FDA had specified the maximum allowable concentration of Hg in human food as 0.5 ppm daily; this is above the natural level of 0.2 ppm. Then in New Mexico the Huckleby family was severely poisoned when its members consumed meat from hogs to which the father had feed surplus seed treated with mercruy fungicides[37,38]. Though action was still delayed, individual investigators in Canada received grants to study the possible existing pollution by mercury. J. A. Keith of the Canadian Wildlife Service pushed through the investigation of fish in Lake St. Clair in March 1970, leading to the discovery of toxic levels. As a result the FDA in Detroit began an investigation, while the Canadians traced the sources and halted commercial fishing in the lake. Two weeks later Michigan acted similarly. The sources were chlorine-caustic soda plants. By the end of April Manitoba had severely restricted fishing and Ohio had closed Lake Erie to American commercial fishing. By the middle of April the FDA had begun organizing a comprehensive study that led to removal of canned tuna from the market, then the discovery of similar levels (exceeding 0.5 ppm) in canned swordfish. This was the beginning of the recent mercury research in the U. S., its development strongly influenced and aided by the Swedes. In a comprehensive special report to the Secretary's Pesticide Advisory Committee, Department of HEW, the study group (members from the committee, FDA, Department of Interior, Federal Water Quality Administration, National

Air Pollution Control Administration, and Department of Agriculture) states as one of their conclusions:

> The current problem can be divided into two broad parts: One relates to the toxicity of unmodified mercury pesticides and the other to accumulations in aquatic systems of alkylated mercury from a variety of sources ...
>
> "The aquatic problem concerns, almost wholly, a mechanism whereby mercury in the bottom muds of contaminated waterways is converted into methyl-mercury which is far more toxic to the brain on a chronic basis than is inorganic mercury[39]."

The toxicity has been studied by many workers since the early 1900's[40,41,42,43,44,45,46,47,48,49,50,51,52,53]. References to studies of the Minimata and similar cases have already been made. Even more work has been done re the analysis of mercury. Dr. Wood is among the leaders re the research into the actual mechanism of conversion. Despite all this the problem still faces us and perhaps is more serious than initially concluded.

B MERCURY: ITS OCCURRENCE AND DISTRIBUTION

The immediate problem is halting the sources of pollution. Yet "existing deposits of mercury in inland waterways constitute a continuing source, through the action of microorganisms in the bottom mud, of the more toxic methyl-mercury[54]". The question of natural occurrence of mercury arises.

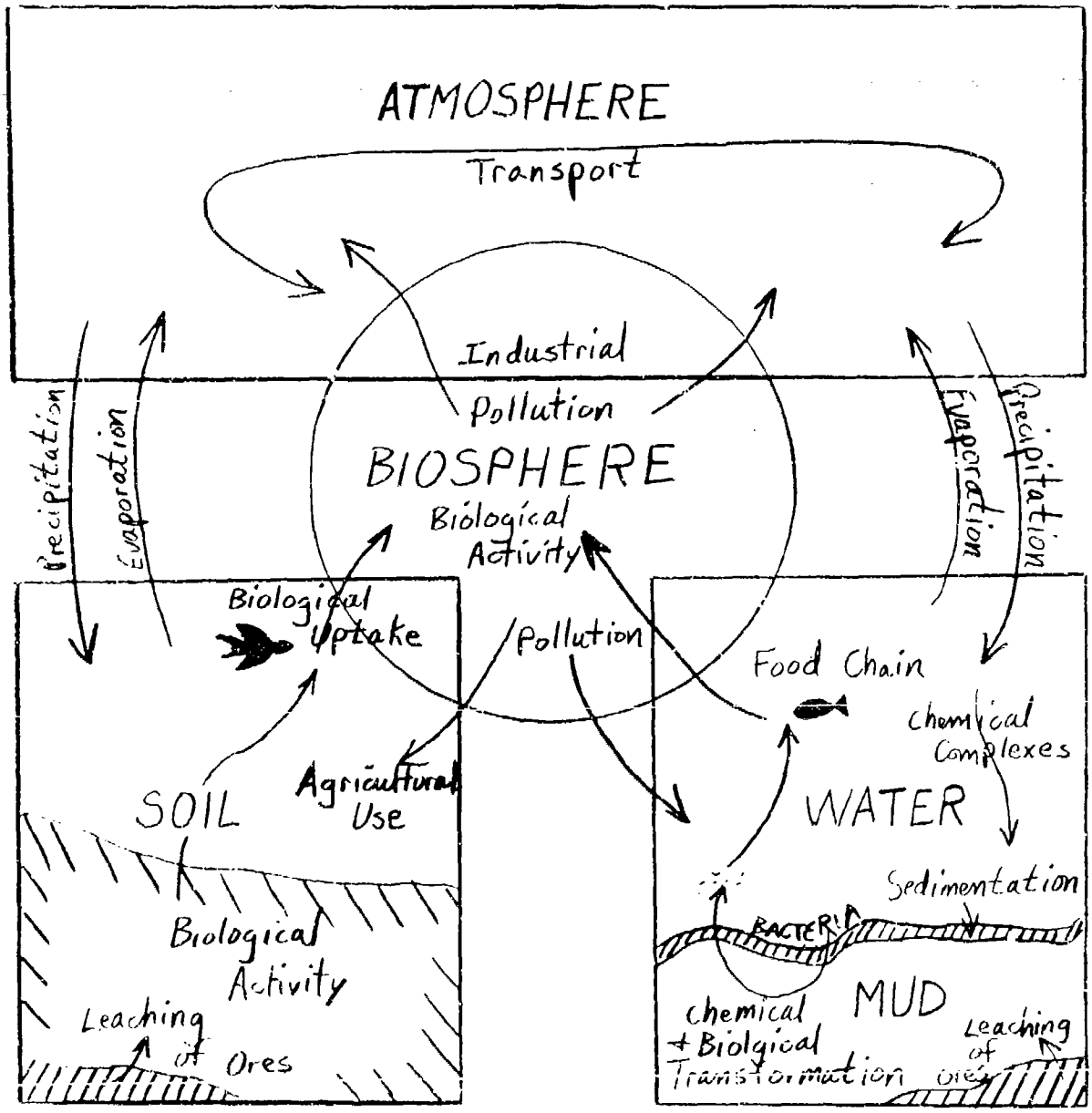
A prominent expert in the field, Dr. Leonard J. Goldwater of Duke University, believes that Hg played a role in most every earth lifeform's evolutionary background[55].

Chemically Hg is referred to as a "noble metal", referring to its stability as an element and also in compounds. This is probably because its valence electrons ($6s^1$) penetrate further into the electron cloud, nearer the nucle than most other metals. A member of the zinc sub-group its most common valences states are +1 and +2, of which the mercuric is the more common. Hg combines with many organic substances to form compounds of which it has been shown that the alkyls are by far the most toxic. Hg(II) usually gives tetrahedral complexes, with a strong preference for large polarizable ligands (such as amino acids and proteins). N-ligands give very stable complexes and chelates but Hg has an even stronger attraction for sulfur. This is the factor in biological uptake already mentioned.

The binding tendency with organic substances is certainly a factor in the distribution of Hg in nature. The ore of mercury is cinnabar, mercuric sulfide. Rocks and soil may contain about 0.1 ppm, usually less, but some shales and fossil organic matter apparently concentrated Hg when living, the effect magnified by the deposition. The Hg content of the oceans, due to the leaching of natural sources, averages 0.1 ppb., making about 10^6 metric tons of Hg in all the oceans. Hg occurs in the atmosphere, both inorganically and organically, the concentration perhaps higher near man-made air pollution sources (burning of fossil fuels). 16 ppb has been found near ore deposits. 0.008 to 0.21 ppb is the normal range, with seasonal trends[56,57,58]. Atmospheric mercury may be due to the

volatility of dimethylmercury and Hg°. Besides the combustion of fossil fuels, chlor-alkali plants may also be a source[59].

GROSS ECOLOGICAL CYCLE OF MERCURY[e]



Though atmospheric mercury content is only roughly known, Eriksson[60] has estimated a concentration which projected globally would give a total of (8) 10^5 metric tons. Man's contribution this century, about 10^5 metric tons[61],

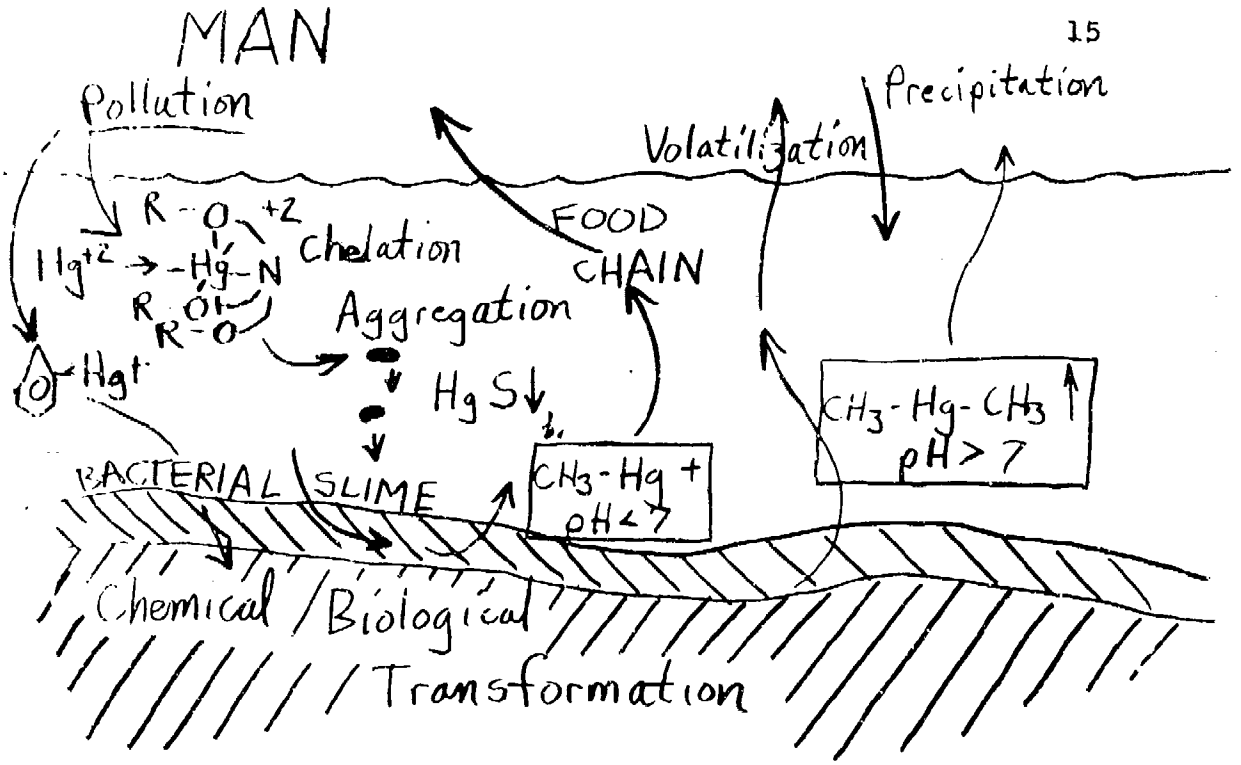
EXPECTED CONSUMPTION OF MERCURY IN U.S.[f]

Outlet	1969		1974-75	
Agriculture	107	TONS	101	TONS
Amalgamation	7		9	
Catalysts	112		89	
Dentistry	116		144	
Electrical	710		863	
Chlor-Alkali	788		869	
Laboratory	78		79	
Industrial Control	265		351	
Paints	370		407	
Paper/Pulp	21		9	
Pharmaceuticals	27		25	
Other	368		226	
Not accounted for	42		na.	
TOTAL	3006		3172	

is in order of one tenth of the atmospheric burden and an order of one thousandth of the oceanic burden. If the average mercury concentration in coal is taken as 1 ppm, the annual use of (5) 10^8 tons would mean 450 metric tons come from this source, not considering contributions from other petroleum processes. Selikoff summarizes these miscellaneous contributions from man, in a partial list as follows[62]:

1. Refuse from hospitals, laboratories, and dental clinics.
2. Disposal of thermometers, barometers, aerometers, relays, rectifiers, switches, fluorescent tubes, mercury lamps, and batteries.
3. Processing or use of raw materials containing mercury such as carbon, coal, chalk, phosphate, pyrite, and so on.
4. Manufacture of and residue from paints and impregnating agents which contain mercury to impact mildew resistance.
5. Refining or redistribution of mercury.
6. Use of mercurial compounds to prevent mildew in commercial laundries.
7. Use of alkali produced in chlor-alkali plant, which may contain as much as 4-5 ppm of mercury.

These, with the major contributions of chlor-alkali discharges, use of mercurial catalysts, seed treatment, burning of fossil fuels, and the use of slimicides (now virtually halted), can be seen to be connected directly or indirectly to the marine environment. Through some use has discontinued, large deposits exist downstream from paper mills and chlor-alkali plants, providing a continuing source to marine ecosystems. It is not unusual for these sediments to run over a range of 1-100 ppm in mercury, or even higher.



MARINE ENVIRONMENT CYCLES

Precipitation increases this content; run-off containing mercury from plant seed treated with fungicides also contribute, but much less than that previously from paper mills and chlor-alkali plants.

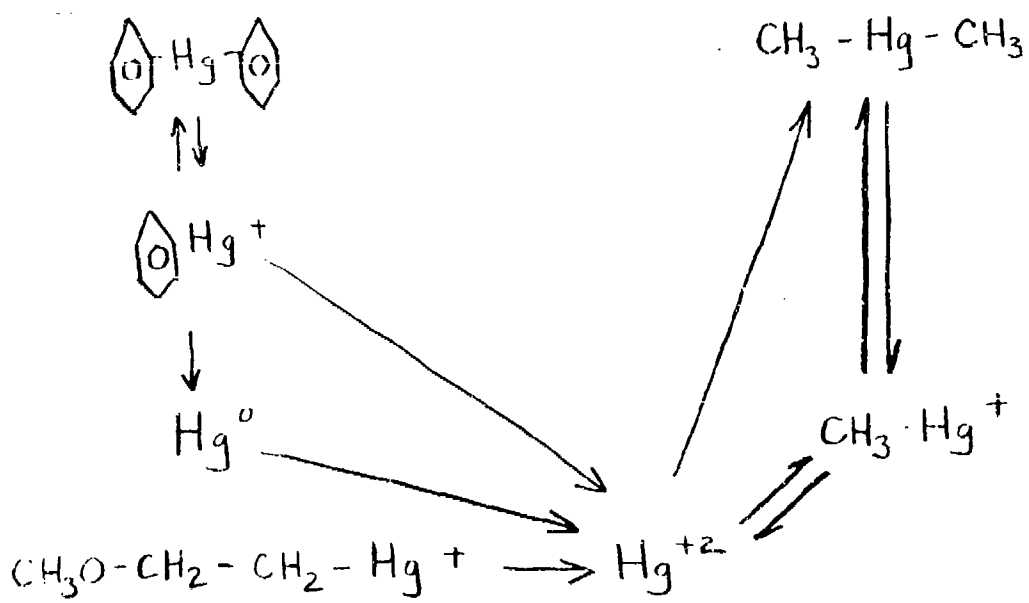
Contribution by the leaching of ore deposits is significant in only a few isolated lakes.

Of all the possible ecosystems, those associated with the marine environment have been most studied. Just after Jensen and Jernelov demonstrated the methylation by sediment micro-organisms in fresh-water aquaria and in bottom sediment from Lake Langsjon near Stockholm[63], Wood, Rosen, and Kennedy[64] isolated and demonstrated enzymatic methylation

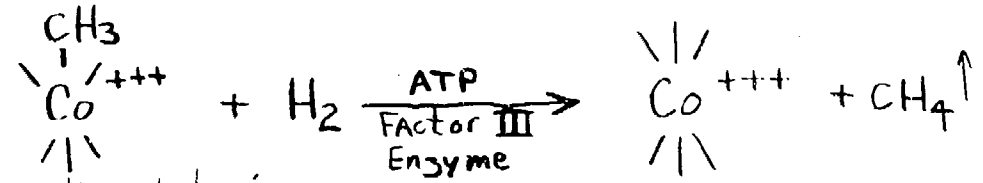
of mercury by a methanogenic bacterium existing in the muds of canals in Delft, Holland. Both mono-and dimethyl-mercury were initial products, depending on pH[65]:

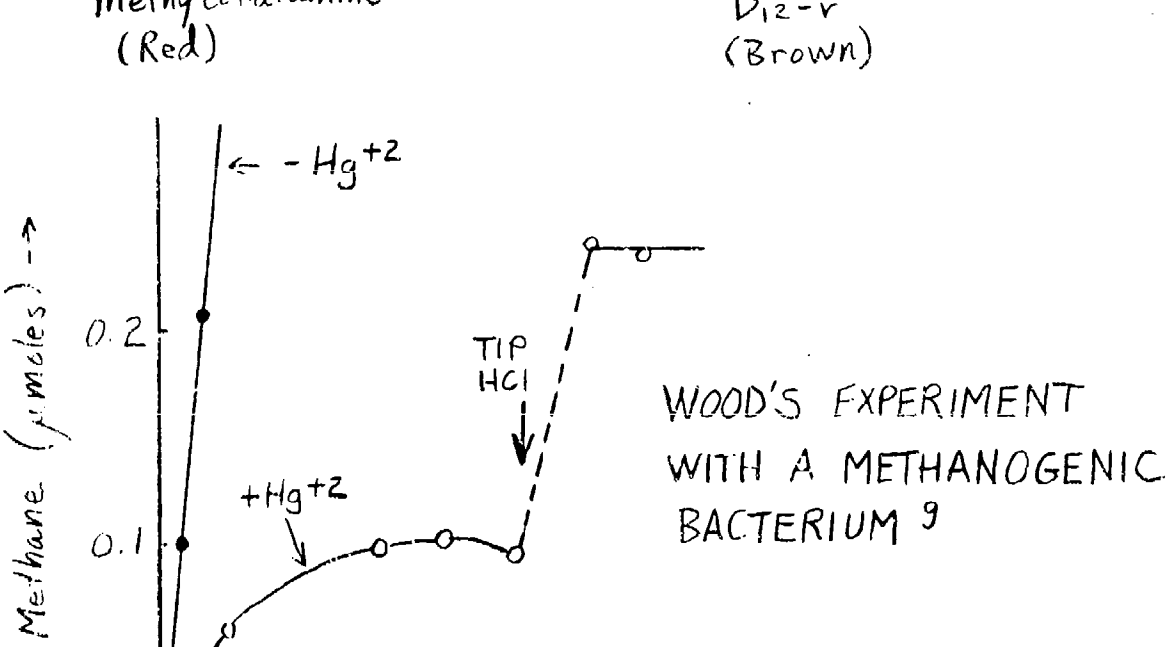
> These two products behave quite differently. The mono-methylated product is directly accumulated by organisms in the water, while the dimethylated form is purported to leave the limnic ecosystem and go into the atmosphere. The most important factor to influence the net outcome of the methylation process is the pH of the water and sediment. High pH values give higher yields of dimethyl mercury. (Olsson, 1968)

Jernelov considered these reactions to be possible[66].



He also believed that the reaction beginning with phenylmercury could be more efficient for the end result, methylation, than direct methylation of Hg^{+2}. Wood's investigation showed the transfer of the methyl group (CH_3°) from Co^{+++} to Hg^{++} was effected in the cultrue of the methanogenic bacterium. He investigated several alkylcobalamines as possible substrates (R - Co --- 5,6-dimethyl benzimidazolylcobamide); in the one producing methane, R = $CH_3^{\bullet}$:





WOOD'S EXPERIMENT WITH A METHANOGENIC BACTERIUM [9]

From the graph, it is apparent that the presence of Hg^{++} inhibited the release of methane; this is due to the formation of Me-Hg and Me-Hg-Me, as was confirmed by thin-layer chromatography. Wood then made use of the reaction

$$R\text{-}Hg\text{-}R' + HCl \longrightarrow \underset{\text{Methane}}{RH\uparrow} + R'\text{-}Hg\text{-}Cl$$

In the case of the introduction of HCl part way through the inhibition reaction, methane was again released as the dimethyl form was converted to the mono-methyl chloride. Methylation of mercury occurred even with small amounts of mercury present. In acid pH, methylmercury was formed despite larger Hg concentrations. This led Wood, et. al. to state[67]:

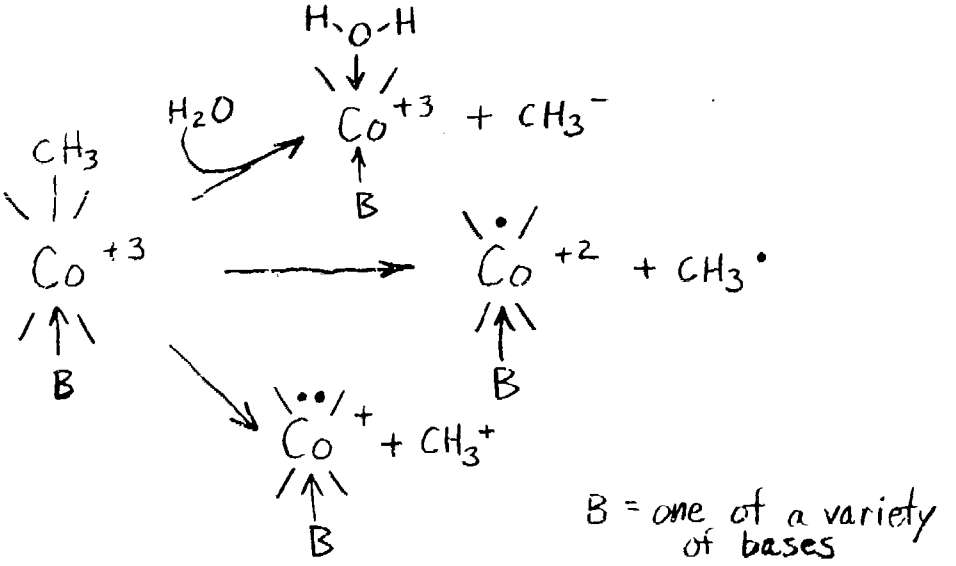
> Acid precipitation of protein is usually used before the extraction of alkyl-mercury compounds into organic solvents. It therefore seems that dimethyl-mercury could be the product of biological significance in mercury poisoning, and methylmercury could be an artefact of isolation procedures.

Wood and Jernelov both investigated the possibility of non-enzymatic process of methyl transfer. Wood, et al.[68]:

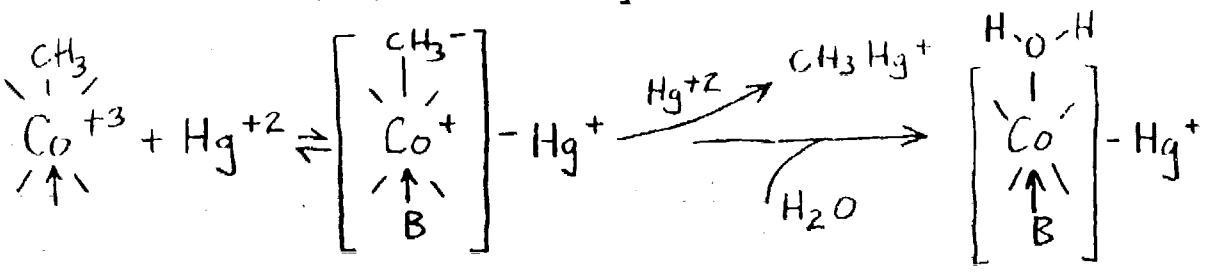
> Apparently transfer of methyl groups from Co^{+++} to Hg^{++} in biological systems may also occur as a non-enzymatic process. If this methyl-transfer reaction is significant in biological systems, then it will be enhanced by anaerobic conditions and by increasing numbers of bacteria capable of synthesizing alkyl-cobalamines. This means, for example, that pollution of a body of water with nutrients (that is, sewage) will increase the rate of formation of methylmercury. Could be formed by both enzymatic and non-enzymatic reactions, thus making this cumulative poison available for incorporation into various organisms in the aquatic environment, and secondarily into terrestrial predators. The cumulative nature of mercury poisoning can be illustrated in fish. The extensive survey performed in Sweden promoted legislation on the use of organomer-

> urials in addition to close control of mercury pollution. Similarly in Japan legislation was brought into effect after the Minamata disaster. We feel that the example set by these two countries should be followed elsewhere before concentration of men research a point where methylmercury is being titrated in humans as well as fish.-

This experiment was performed at least a year before such legislation was effectively begun. Wood, a biochemist, has since done further research into the mechanism. "Of the three major coenzymes known to be available for methyl transfer reactions in biological systems - S - adenosylmethionine (SAM), N^5-methyltetrahydrofolate derivatives, and methyl corrinoid derivatives - the first two involve methyl group transfer as a carbonium ion. Therefore, Dr. Wood concludes, only the methyl corrinoids are capable of methyl transfer; since they are theoretically capable of transferring methyl groups as carbanions (CH_3^-), carbonium ions (CH_3^+), or radicals ($CH_3^\bullet$)[69]."



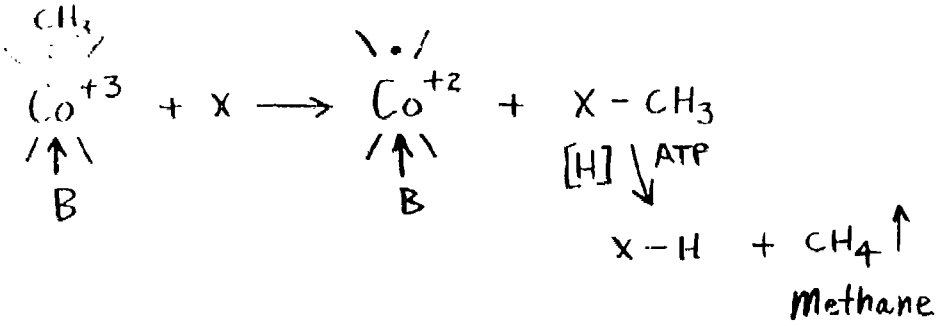
Further research led Dr. Wood to suggest this reaction series for the transfer to mercury:



"This nonenzymatic reaction does not occur in the presence of Hg^{+2} or Hg°. Therefore, Dr. Wood concludes, this reaction would be predominant in those aerobic organisms that use methylcorrinoids in their intermediary metabolism, since under anaerobic conditions $2Hg^{+2}$ plus two electrons yields Hg_2^{+2}, and Hg_2^{+2} plus two electrons yields 2Hg°"[70].

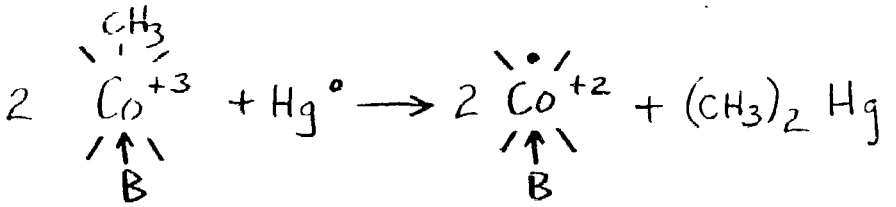
In effect he has described methyl transfer (as the carbanion) in acidic, oxidizing environments (where methyl corrinoids are used in aerobe metabolism) with special dependence on the second mercuric ion.

Enzymatic methyl transfer may occur in aerobes and anaerobes in connection with methionine synthesis, "as well as in mammalian liver"[71]. Dr. Wood lists two additional enzymatic sources: anaerobes capable of synthesizing acetic acid, and the more common anaerobic methane synthesis, in which Dr. Wood has shown that the methyl group is transferred as a radical. The normal series for methane synthesis,

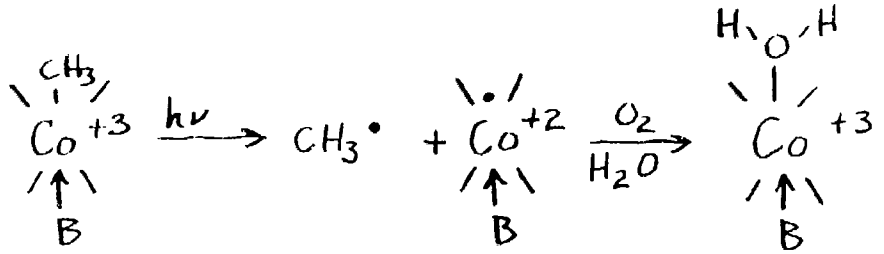


X = unknown substrate

could be interrupted by the presence of inorganic mercury because the growth conditions (redox potential) of the anaerobes is such "any inorganic mercury salt added to the methane synthetase enzyme system would be reduced to Hg°, Dr. Wood finds. Dimethylmercury is then synthetized by CH_3 addition to Hg°!"[72]

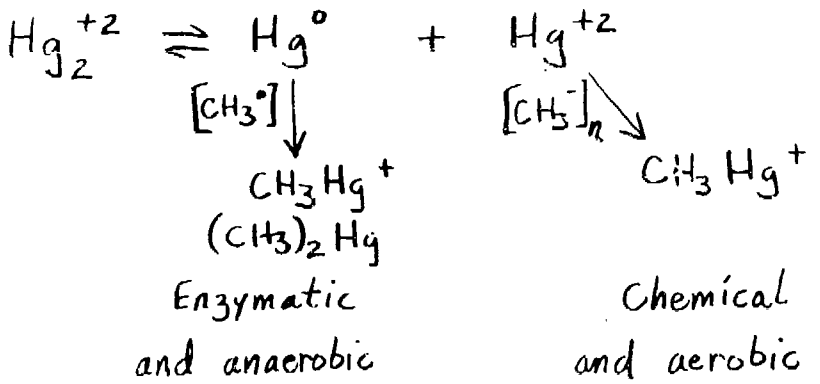


where the CH_3° could be made available by photolysis:



After experimentally confirming the feasibility of such reactions, Dr. Wood concluded "that Hg^{+2} may be transported across cell membranes by these anaerobes, reduced to metallic mercury, and then methylated..."[73]

If the sediment is acidic the dimethyl form would be converted to the monomethyl form as was shown by Wood's experiment in 1969. Dr. Wood believes "methyl corrinoids are present in a synthesis of methylmercury compounds in both aerobic and anaerobic systems .... Therefore, the rate of synthesis will be dependent on the population of microorganisms in these ecosystems"[74].



The pollution of our marine environment with nutrients (carbon containing compounds, nitrates, phosphates would thus lead to overproduction of such microorganisms, as Dr. Wood indicated. The results of some experiments have given support to this[75,76].

Thus methylmercury may be synthesized from mercury pollutants in sludges and then released for uptake by flora and fauna occupying niches in these ecosystems. A possibility of methylation within the microbial systems of fauna also exists[77,78,79,80]. Because of the complexity and feasibility of methylmercury synthesis under varying environmental conditions, it could occur to some extent wherever mercury pollution exists though it is probably more efficient in aerobic systems. Halting the conversion would require elimination of the multitude of microbial methylating systems ("starve them" as Dr. Wood has said), or someway of removing the whole section of polluted mud from the waterway (dredging, etc.). Covering them with an inert substance (such as fluospar tailings) has been shown to be both theoretically and experimentally unsatisfactory, though it slows down release of Me-Hg, which is held loosely by the sediment[81]. This would be satisfactory for a while. If active heavy metal absorbers were used, i.e. quartz and silicate minerals with open lattices, as shown by Landner[82], the mercury would still be there for later availability if the conditions change (One other possibility exists. If the oxygen content is very low, the mercuric sulfide could be formed which is harder to methylate[83]. Thus alkali lakes, where the eutrophic condition makes H_2S available, might precipitate HgS to unavailability because of the inactivity the mineral, etc.). But natural processes such as turnover

by bottom dwellers would eventually redistribute underlying mercury. The main advantage to covering would be a temporary halt while research forces were gathered for developing a more efficient solution.

Just as mercury pollutants can be transported by chemical, physical, and biological processes to bottom sediments (e.g. chelations, absorption into organic aggregates and eventual settling, biological recycling through death, diffusion, movement of polluted water,), the loosely bound Me-Hg, or Me-Hg complexes/chelates, is available to the food chain where it can be concentrated much more rapidly than other mercury forms, as has been indicated. However, Hannerz (1968) has shown the normal food chain concentration at the peak of the pyramid may be reversed according to metabolic rates and food habits.

Where streams move rapidly the transport of the Me-Hg or pollutants themselves may lessen the concentrated effect existing near mercury deposits. Release by voltalization of Me-Hg-Me may also reduce the danger. However, the mercury compounds are still made available elsewhere; it would require most existing deposits many years to be significantly reduced.

Fish are the major food source of mercury poisoning for man the predator. Marine animals may absorb the mercury directly out of the water or indrectly from their food[84]. Different species, different sex, different age and size, different migratory habits, etc. account for variations in

Me-Hg uptake, but in general the elimination is so slow that dangerous levels build up.

Fish from Minamata and Niigata were on the order of 10-20 ppm, wet-weight, while the average of pike analyzed in Sweden by Miettinen, et. al.[85] was onthe order of 6-7 ppm. These concentrations are sufficient for observable sypmtoms of mercury poisoning. The absorption through the gills can be significant at concentrations of the surrounding water on the order of 0.1 ppm[86]. Reduction of photosynthesis of plankton by oranomercurial fungicides concentrated from 1-10 ppb have been observed in the laboratory[87]. The recent FDA analysis of swordfish and tuna showed that few fish contained more than 1 ppm, while most of the tuna averaged from 0.13 ppm (smaller than 12 kg) to 0.25 ppm (more than 23 kg)[88]. Thus various marine life are affected by mercury in the water, from plankton at ppb levels to large fish at ppm levels. However, fish containing levels over 10 ppm are vary rare, the vast majority averaging much less than the FDA limit of 0.5 ppm. Consumption of polluted fish would yield disastrous results if eaten on a regular daily basis for several months. The level of fish from unpolluted waters runs less than 0.06 ppm, offering little danger, while the swordfish and tuna restricted by the FDA could have caused isolated cases of mercury poisoning.

Animals from other environments can be connected to the problem either directly (flocks of birds eating recently planted treated seed) or indirectly (predator birds and

mammals consuming polluted fish, mussels, etc.). Sweden first suspected pollution of her environment when flocks of seed-eating birds were noticed to be diminishing in size. The effect of poisoning in mammals has been demonstrated only too well by man himself. As Westoo has shown the most mercury in foods is Me-Hg, a human contacts this toxic compound directly when he consumes polluted food.

SOME TOTAL MERCURY AND METHYL-MERCURY CONTENTS OF SWEDISH FOODS IN 1966[h]

Food	Total Hg (ppb)	Me-Hg (ppb)	% Me-Hg
Meat (Ox)	74	68	92
Meat (Poultry)	23	17	74
Liver (Pig)	130	95	73
Liver (Pig)	96	75	78
Egg yolk		5	50
Egg yolk		9	90
Egg white	2	11	92
Egg white	25	24	96
Muscle tissue (perch)	220	200	91
Muscle tissue (perch)	3250	2990	92
Muscle tissue (pike)	3350	3110	93
Muscle tissue (pike)	560	550	98
Muscle tissue (cod)	64	55	86
Muscle tissue (cod)	26	22	85

A closer look at what is known medically about mercury poisoning regarding the human being can give final emphasis to the seriousness of the mercury pollution problem.

II. Mercury Poisoning

People become concerned about the problem of pollution mainly when they can see that it can affect their livelihood in some way. Although cases of mercury poisoning are relatively few and isolated, the dramatic, tragic results always awakens some interest, and recent events and findings have shown that it can extend to a generally public nature, e.g., the Hukleby incident in New Mexcio[89] and the findings of mercury in tuna and swordfish in the U. S.. Outside of these cases and those of Minamata, Niigata, and the few other similar incidents, most of the other reported cases are "direct contact" cases, i.e., where people absorbed the toxic compounds directly. These, documented as referenced above, have provided much in the form of a contribution to medical knowledge, but really have little to do with the long-term mercury problem. The problem that faces us as concerned humans is the mercury, that we find in our food, occasionally at "toxic levels". This has been shown to be most often methyl-mercury, the seriousness of its poisoning has been previously mentioned. This originated at the other end of the food chain with a biochemical transfer of a methyl group to an inorganic mercury ion, which happened to get in the way of the normal methyl transfer, which is involved in many of the biological processes of micro-organisms. Since mercury exists at natural levels we are not responsible for making the conversion possible

in the vast majority of ecosystems. Where we have allowed large beds of mercury pollutants to build up, whether accidentally or deliberately, with or without the excuse of ignorance, is our fault and is what makes for potentially toxic levels in our food. Though we have contributed less the 1% of the natural burden of mercury by mining, processing, and use since 1900, we have concentrated much of it in local areas, giving rise to that conditional presence of the inorganic mercury ion to interrupt a natural methyl transfer. Though the knowledge of mercury poisoning is largely from case studies of agricultural, industrial, and laboratory accidents, the significance is directed toward the possibility of the appearance of symptoms from the uptake from chronic consumption of polluted foods.

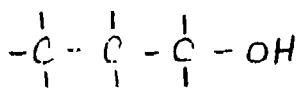
Methyl-mercury is 100 times more toxic than inorganic mercury, yet the quantity or concentration to be considered "toxic" has not really been defined. Dr. Goldwater says

> Furthermore, high levels in the urine or the blood do not necessarily indicate poisoning, many cases have been observed in which the individual had a mercury concentration in the blood amounting to 10 to 20 times the "normal" upper limit and yet, showed no indications of illness or toxic symptoms! All in all there is substantial evidence that host factors may be more important than the amount of exposure, up to a point, in determining the individual's response to mercury in the environment. In any case, urine or blood analysis is of no value for early diagnosis of mercury poisoning. Other possible indicators, such as disturbances of the blood enzyme system, are being investigated, but no reliable diagnostic test has yet been produced. Nor can we define a precise threshold for the toxic level, either for exposure to or for absorption of mercury[90].

The symptoms of inorganic mercury poisoning derive from its action in the digestive tract. The symptoms of organic mercury poisoning are neurological in nature. The patient may at first notice some loss of sensory preception, loss of memory or ability to think clearly, indicative that the presence of organic mercury in the nervous system has affected, probably destroyed, all the paths of redundancy within the system. That is, up until this point the brain has been able cope with destruction by compensating with other pathways. This may occur at blood levels of .2 ppm or brain levels of 3-20 ppm, varying largely because of host nature. The strong binding of methylmercury with thiol groups is responsible for the brain's, the liver's, and the body's small half-life of elimination. Decomposition and deposition of the organic mercury compound as other forms may also be a factor in the slow elimination of methylmercury. Measured half-lives are 50 days for the liver, 150+ days for the brain, and 70 days for the body considered as a whole[91]. Simple extrapolating from these data, assuming a daily intake of 100 micrograms (from moderately polluted fish), show that, though the brain content builds up slower, it does not level off until long after the point of death which in this case would happen in a matter of months.

The important fact is that, since brain cells are irreplaceable and perhaps 20% of the methylmercury is absorbed and found in the brain, damage will occur even if symptoms

do not appear. If symptoms appear, partial recovery is possible if poisoning is not any more severe. In severe cases, i.e., where enough has been absorbed (unknowningly) to exceed the "symptom level", the damage may progress to death. In between, the symptoms progress to complete blindness, loss of coordination and rationality; convulsions and diarrhea are common towards the end. Sedatives are not effective and the use of chelating agents do not have any definite effect (their hopeful use is in binding with the mercury compound in an attempt to flush it out in elimination). The best known chelate is BAL:



2,3-dimercapto-propanol

but it in itself is toxic and requires careful administration[92]. Ca-EDTA (salt of ethylenediamine tetracetic acid) caused higher mortality in control animals[93] while penicillamine (D or DL forms) seems to be effective in mercuric chloride poisoning. Even more effective, n-acetyl-DL-penicillamine is less toxic[94]. Prick, Sommen, and Slooff have suggested the use of artifical kidney to filter out the compound[95]. Atopsies have shown visible brain damage[96] and chromasomal damage has been discovered in persons after consuming methylmercury containing fish, concurrent with high blood levels of mercury[97]. There is also evidence that the mercury compound can be transferred across the placental barrier[98]. Outside of such forboding findings, organic mercury compounds can cause second degree burns upon

contact[99] and are even more easily taken into the body by breathing than by being absorbed through the skin (which is particularly easy for organic compounds in general). The people in the United States carrying around the 230,000 pounds of inorganic mercury in their mouths as amalgams in their dental fillings[100] need not be concerned because Hg° is dissolved so slowly (to Hg^{+2}) that toxic inorganic levels could never be reached, as findings have verified. The laboratory workers who use organic mercury compounds as standards and reagents had on the other hand better known exactly what they are doing. The public in general should be concerned, certainly, but as Dr. Goldwater concludes in his article in Scientific American[101];

> It would be foolish to declare an all-out war against mercury. The evolutionary evidence suggests that too little mercury in the environment might be as disastrous as too much. In the case of mercury, as in all other aspects of our environment, our wisest course is to try to understand and to maintain the balance of nature in which life on our planet has thrived.

III. MERCURY ANALYSIS

Since mercury can be found in so many different materials in different forms and concentrations, applicable analytical methods vary from one type to the next. A partial list of samples includes

(1) various tissues, urine, blood of fish, humans, etc.
(2) all types of foods, processed and improcessed
(3) air and water samples from different areas, e.g., effluents waste runoff
(4) soils, sediments, sludges from waterways, dumps, sewage treatment plants
(5) industrial products and wastes

Some of the analytical properties of mercury compounds are:

(1) voltatility
(2) extremely strong absorption lines, especially the one at 2537Å(ultra-violet)
(3) strong binding with sulfur groups
(4) radioactivity of its isotopes primarily

Hg-203 (half-life = 47 days) and

Hg-197 (half-life = 65 hours).

The primary analytical methods are

(1) dithizone extraction and photometry of the complex
(2) benzene extraction/cysteine partitioning and gas chromatography of a final benzene layer
(3) atomic absorption (flame)
(4) atomic absorption (flameless)
(5) neutron activation analysis.

Each method involves most or all of the following aspects:

(1) sampling
(2) isolation of desired Hg-compound(s) and/or
(3) digestion of the sample
(4) converting the Hg-compound(s) to a detectable form or getting it into a suitable solvent.
(5) instrumental procedures.

The oldest method is that involving dithizone extraction. The sample was acid-digested the mercury (in the Hg^{+2}, oxidized form) complexed with dithizone (diphenylthiocarbazone), and the absorption intensity of the complex measured. Its approximately 0.5 ppm sensitivity (under favorable conditions) is not of the order desired for modern analysis (0.1 ppm or less, preferably on the order of 0.001 ppm). Greater sensitivity can be achieved (to 0.000005 ppm) by sample concentration, which is applicable only to water samples and susceptible to loss.[102]. Volatilization of Hg compounds during the digestion-oxidation process also gives rise to loss of Hg. The extraction procedure has been applied to organo-Hg(II)[103]. Among the variations of the dithizone method, that of Chau and Saitoh combines the extraction with flameless atomic absorption, with a sensitivity of .000008 ppm for water samples.

Atomic absorption involves either excitation of Hg in a solution spray by a flame and the absorption of the characteristic line from a (usually) monochromatic beam from a mercury lamp, or reduction-volatilization of Hg^{+2} in acid solution, passage of the cold vapor through a long-path absorption tube, and measurement of the absorption of the characteristic line emitted from a mercury lamp[104].

The former is relatively insensitive (100 ppm). The latter is a very rapid method of determining total Hg to concentrations as low as 0.002 ppm. Many workers have developed it[105,106,107,108], and it is applicable to all types of samples. By varying sample treatment, organic mercury forms can be separated and determined[109]. It has been widely adapted in the U. S. and Canada because of its inexpensiveness, rapidity, and sensitivity. In fact, most of the time involved is expended in sample treatment, the analysis taking only a matter of seconds. The sample is first acid-digested, usually with a combination of nitric and sulfuric acids, which also provide oxidation. Further oxidation is carried out by addition of potassium permanganate and potassium persulfate; room temperatures or below are used to minimize loss by volatilization (some occurs despite the oxidation, hence the necessity for running recoveries, i.e., standard spiked samples carried through the same procedure). Excess $KMnO_4$ is neutralized by hydroxylamine hydrochloride. The sample is then attached to an aerator in a closed system containing the absorption tube, and the Hg^{+2} reduced to Hg° by $SnCl_2$: aeration volatilizes the Hg° and carries it through the absorption tube in a cycle returning to the sample container. The absorption reaches a maximum when the Hg° has been completely evaporated. The value of the absorption (or % T) is applied to a calibration curve prepared from standards spiked with known amounts of mercury.

Dr. Gunnel Westoo (Sweden) has probably contributed more to the development of gas chromatographic mercury analysis than any other worker[110,111,112,113,114]. Although GC does not analyze Hg^{+2}, with proper columns and detectors it gives individual peaks for many organo-Hg compounds, among them mono and di-methyl mercury. It is the toxicity of methyl-Hg and Westoo's discovery of it presence in fish and foods that gives this analytical characteristic importance. GC analysis for methylmercury is difficult but experienced analysts with proper equipment may develop sensitivity to 0.001 ppm and precision to 12%[115]. Samples are homogenized, acidified with HCl, and extracted as the chloride into pesticide-grade benzene[116]. The benzene layer is removed and partitioned with a 1% cysteine solution, which is removed, acidified with HCl, and extracted with benzene. The final benzene layer is analyzed by GC. Several columns are useful[117,118], and an electron capture detector is required. Because of sensitivity to contamination a check of all reagents used must be made.

More elegant but more expensive and involved is the neutron activation method of analysis:

> In 1964, a great improvement in sensitivity to about 0.001 ppm in an 0.5 g sample was achieved (Sjostrand, 1964) by introduction of chemical separation steps. Thus, after the neutron irradiation (2 or 3 days at a thermal neutron flux of 10^{12} n/cm^2 per second) the sample is decomposed by boiling with nitric and sulfuric acids in a closed system, and 20 mg of carrier mercury are added as mercuric chloride; then, perchloric acid and glycine are added and the mercury is distilled off at temperatures up to 250° as a volatile compound.

> Next, the mercury in the distillate is electrodeposited into gold foil, the foil is weighed, and the 197 Hg content is determined by gamma-spectrometry with a sodium iodide crystal and 200 channels of a multichannel analyzer, appropriate corrections being made for the percentage of recovery of the added carrier. This method has a precision and accuracy of about ±2%, and is generally regarded in Sweden as the preferred method for determining total mercury in biologic samples. (Greater sensitivity is possible through use of a high neutron flux)[119].

Other analytical methods include mass-spectrometry and thin-layer chromatography used by Westoo to identify (in addition to the use of gas chromatography) the methyl-mercury in biologic materials. (Westoo, 1970,1967,1966).

A relatively little used method is that of emission spectrophotometry in a radio frequency plasma[120], which is supposedly sensitive to the presence of $(2)10^{-9}$ g of Hg. "The technique is basically that of flame photometry with the flame replaced by a radiofrequency plasma in helium at atmospheric pressure. The high emission sensitivity of mercury in the helium plasma and extremely efficient system for extracting the mercury from the sample in a small volume of helium gives the method its striking advantages[121]."

IV. RESEARCH PLANS

A. *ORIGINAL PLANS*

During the spring semester of the 1970-1 academic year, in research course SC492, a literature survey of mercury pollution was prepared in preparation for Trident Research during the 1971-2 academic year. In addition to the preparation of a list of 142 references, a plan of research was outlined. It was proposed to study two general problems: (1) the modeling of laboratory environments containing a sediment and an indicator species of fish, in which the factors to be studied would be controlled; and (2) the analysis of the indicator fish for methyl mercury uptake.

The factors to be studied included intially:

(1) sediment/bacteria type and activity
(2) fish as indicators
(3) pollutant-type and concentration
(4) time of conversion
(5) presence or absence of a chelator
(6) acidity (pH)
(7) oxygen content of environment
(8) temperature of environment

The sediment types to be studied were: (1) a sediment that had been polluted while in a waterway, which would be furnished by a commercial chemical company; and (2) a formerly non-polluted sediment which would be polluted in model environments with inorganic mercury and organic mercury compounds, which were similar to mercury compounds used in agriculture and/or industry.

Goldfish were selected as the indicator fish for two primary reasons: (1) they are related to pike, which have been shown to uptake methylmercury well; and (2) they are inexpensive, easily obtained, and relatively easy to maintain. Their livers were to be analyzed as short-term indication of methylmercury uptake.

The inorganic mercury would be added as mercuric chloride ($HgCl_2$) and the organic mercury compound would be the phenyl mercury radical, to be added as the nitrate or acetate. The concentration were to be 10 ppm and 100 ppm re the sediment.

The times of conversion were to be at selected intervals up to one month. Acidity would be monitored as the pH of the water above the sediment, whose volume would be compensated for loss by evaporation. The temperatures at which conversion rates were to be studied were selected as 0°C (refrigeration), 25°C (room temperature) and 55°C (oven).

The two standard methods used for mercury analysis are gas chromatography (GC) and atomic absorption (AA). The first method is used to determine methylmercury; the second total mercury. Because of the problems involved in gas chromatography which include obtaining and conditioning a suitable column and obtaining and installing an electron-capture detector, which required the use of tritium foil and the obtaining of a permit for the use of tritium, a

portion of this analysis would be carried out elsewhere[b]. Thus it was proposed that the sampling, digestion, extraction and partitioning to obtain the final benzene would be done in our laboratories and complete at the FDA Laboratories.

Likewise since the atomic absorption unit had not been set-up, a portion of this anlayses would also be carried out elsewhere[a]. The original sampling and acid oxidation would be done in our laboratories.

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[a]Dr. Don Lear and Mr. O. Villa of the Environmental Protection Agency, Parole, kindly permitted the use of their Perkin-Elmer (Coleman) MAS-50 unit, which has been automated. We are very grateful for this assistance.

[b]Mr. L. Kamps, Pesticide Division, Food and Drug Administration, kindly permitted the use of his apparatus, for which we are very grateful.

B. MODIFIED PLANS

1. USE OF FISH - As soon as work on the modeling and analysis design began, it was seen that modifications would be necessary. The commercial chemical company informed us that they could not make the polluted water-way sample available. It was then proposed to prepare a polluted sample by the addition of controlled pollutants to non-polluted samples.

A non-polluted sediment was obtained first for three aquariums from a brook emptying into the Magothy River, but these sediments were discarded because of the danger of inactivation of the microorganisms by improper sampling and a technical problem in keeping the sediment on the bottom of the aquarium (both the aeration and the goldfish kept the sediment particles dispersed in the water).

Subsequently, on the advice of Dr. Lear, sediment sample from the Rocky Gorge Reservior (near US 29, north of Laurel) was obtained and used throughout the remainder of the research. A coarse mesh of rubber mat kept the sample down and did not permit its disturbance by fish or currents. Bacterial growth, especially in the 0.5 cm top layer, was tremendously accelerated by wasted fish food and this was visible in a matter of days.

When the sediments were stable, the pollutants were added as their salts directly to the aquaria water in amounts calculated to give 100 ppm Hg re the sediment. All fish died within 15 minutes after the addition of mercuric chloride. Fish were removed from the second task before addition of phenyl mercuric salt, but as soon as added, they died in that tank also. In both cases, deaths occurred as a result of mercury intake for exceeding the toxic level of inorganic mercury and organic mercury. Analyses of three fish from the inorganic polluted aquarium showed that they absorbed 315.4, 364.4 and 459.7 ppm total Hg respectively from water at a total Hg level of 117.6 ppm. The water from the organic polluted tank was only 16.2 ppm total Hg, indicating that only about 10 percent of the phenyl mercuric salt had dissolved.

Water samples and sediment samples were also taken and analyzed but high reagent contamination caused high blanks and, therefore, inconclusive results.

Even though the sediments were left standing for a week (in the hope that the pollutant might be absorbed, resulting in lower and bearable Hg concentration in the water), fish died as quickly as before when placed in the aquaria. The fish died even after the aquaria were flushed out and the water above the sediment replaced three times. Furthermore, it appeared that even if the Hg concentration in the water were lowered enough, the fish would still stir up the sediment sufficiently to disperse absorbed pollutant. Therefore, the decision was made to modify the project to analysis of water

and sediment samples for detecting the production of methylmercury, thereby eliminating the fish and thereby the uncertainties due to biological difference amount the fish.

B. MODIFIED PLANS

2. USE OF SEDIMENT AND WATER SAMPLES

(a) Sediments Aquariua were setup with Rocky Gorge sediment as follows:

Aquarium I - approximately 150 ppm Hg^{+2} and 150 ppm phenyl Hg^{+}

Aquarium II - approximately 150 ppm Hg^{+2}

Aquarium III - approximately 150 ppm phenyl Hg^{+}

In addition, there were also set up testtube (approximately one gram) sediment samples, polluted to 100 ppm by Hg^{+2}. Five samples each were incubated at 0°, 25°C, and 54°C to study the effect of temperature. These set-ups were made so as to determine if measurable methylation would occur with such a small amount of sediment. Jensen and Jernelov used one gram samples.

(b) Analyses - A method of analyses feasible for our laboratories was developed. The new analytical method combined the G C. method and the A.A. method. It was designed and tried successfully with Me-Hg spiked water samples (in varying Hg^{+2} backgrounds) by Ross, Gonzalez, and Moore. It had not been applied to organic samples.

It was decided to incorporate this method into the project, first reproducing the results achieved by the original workers, then to work out the problems it applying in to sediment analysis. It basically involved the GC procedure up to the cysteine layer which was then acid-oxidized and treated by the AA method as a normal sample. It would thus analyze for alkyl-Hg (i.e., methyl-Hg) but would not identify the compound (as would be possible by GC). The chemistry of the extraction, if done without error, would be the only identification (indirect) available.

Practice calibration runs of Hg^{+2} standards were made and Beer's Law plots obtained on the new instrumentation. When the proper reagents arrived, the extraction, analysis, and other type runs were begun in our laboratories.

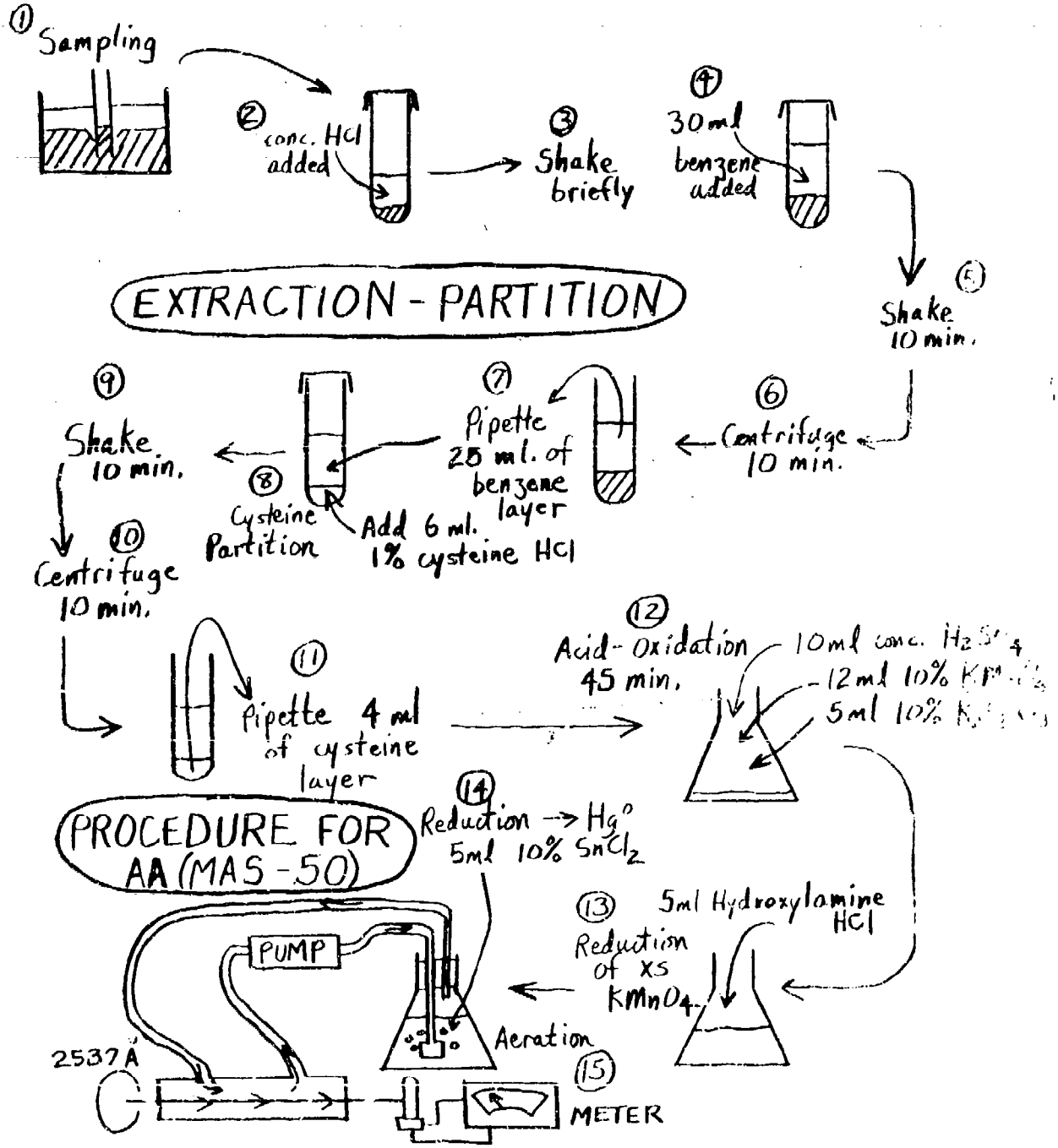
3. PRELIMINARY CHELATION EXPERIMENTS

An investigation into the determination of the formation of various chelates with EDTA (ethylenediaminetetracetic acid) after the pH-monitoring and titrating method of Schwartzenbach was being made to study EDTA's possibilities re the project and to try a method of determination of chelate formation applicable to chelators containing acid groups (This was done as a project in SC401, Instrumental Analysis). EDTA was found to be a weak chelator of mercury and the method inapplicable except in cases where the stability constants of the metal chelates were high enough and the formation of it rapid enough to be determined by automatic titration. The method could have been used, titrating normally after assuring that

solution equilibrium had been achieved, but would have been too time-consuming and probably unrealistic when applied to sediment environments. Instead, the relatively untried but promising chelate NTA (nitrilotriacetic acid) would be added, in a manner conceivable to commercial use, to aquariums polluted with mercury, and analysis of Me-Hg production used to determine its effectiveness in reducing the availability of the inorganic ion in methylating reaction. It was realized that the situation was much more complicated (e.g. NTA might transport Hg^{+2} to positions in the mechansims impossible without the chelator present, and perhaps increase availability thereby), but simplification was necessary.

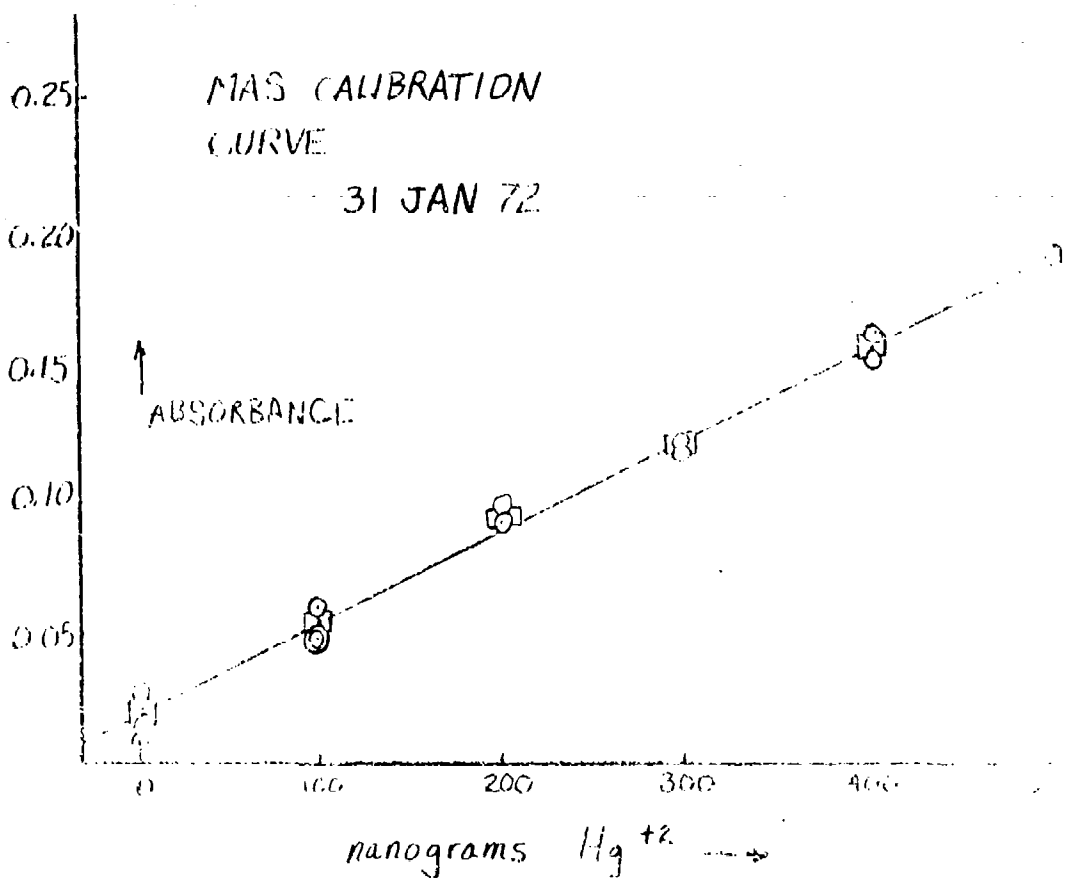
V. EXPERIMENTAL PROCEDURES

The mercury analyzing system procedure which was developed is presented in schematic form.



(1) Extraction Run #1

Prior to this run a calibration curve for the MAS was prepared (one is almost every time an analysis is planned, the instrument is left on). This is a typical curve:



The reagents to be used for the project were checked for mercury content.

10ml	HCl	(12N acid)	<50 ng	Hg^{+2}
10ml	H_2SO_4	(conc. acid)	<50 ng	Hg^{+2}
10ml	HNO_3	(conc. acid)	600 ng	Hg^{+2}
1ml	$KMnO_4$	(10% sol'n.)	<50 ng	Hg^{+2}
1ml	$K_2S_2O_8$	(10% sol'n	<50 ng	Hg^{+2}

As a result of these analyses, a new stock bottle of HNO_3 was selected. Carrying out the procedure for water and sediment, as depicted in the schematic above, the blanks generally ran around 50ng/sample analyzed and this value was subtracted from data obtained (blank correction).

To try out the basic total Hg analytical procedure with the new MAS-50, 100 ml of James River water was treated and analyzed, giving a concentration calculated as .006 ppm which is about normal. Tap water usually gave blanks lower than this, but distilled water was used for dilutions (in some runs, deionized distilled water was used, but the blanks ran about the same, because of high reagent content, so ordinary distilled water was used for the most part).

Samples of water were spiked with 0, 1, 2, and 4 µg Me-Hg, and samples of Control (Rocky Gorge) sediment were spiked with 2 and 4 µg Me-Hg and carried through the extraction procedure (see schematic above). Blanks ran through normal analysis procedure gave 40 ng background. When the extractions were analyzed, small amounts of benzene residual in the cysteine layer blanketed results (benzene has an absorption peak around 2537Å). Future runs employed the aeration of samples (to eliminate residual benezene) before attachment to the MAS. The standard 1 ppm solution of Me-HgCl was checked by oxidation-analysis and found to be actually 1.3 ppm. Some modifications were made to the apparatus at this point.

(2) Extraction Run #2

This run was essentially a duplicate of the last run, except that a double extraction (to increase recovery) by benzene was tried.

Results were also poor. Me-HgCl standard check again gave 1.3 ppm. Blanks on the calibration curve contained 50 ng.

(3) Extraction Run #3

Water sample spikes analyzed using Spectroanalyzed grade benzene: cleaning methods were responsible for poor results. Aeration henceforth was done by a separate air pump to remove residual benzene. Me-Hg check gave 1.5 ppm.

(4) Extraction Run #4

Duplicate water samples were spiked with 0, 0.2, 0.5, 0.8, and 1.0 µg MeHg. The water recoveries gave the first linear curve in the extraction runs. Sediment recoveries were unsatisfactory because the centrifuge did not break the emulsions in the benzene partition effectively. At the risk of somewhat lower recoveries, it was decided to proceed with a single benzene extraction rather than two at half-volumes. Me-Hg check gave 1.6 ppm. Benzene used here and henceforth is Pesticide grade quality. The apparatus was modified so that the sample would be less able to be pumped.

(5) Extraction Run #5

Control sediment was spiked with 0.0 µg (two samples) and 0.5 µg (three samples) Me-Hg. Water was spiked with 0.0 and 0.5 µg Me-Hg. A single water sample was spiked with 0.5 µg Me-Hg and 100 µg Hg^{+2} to determine effect of background pollution. The results were the first reproducible recoveries for sediment samples, though blanks still ran about 50 ng. The water Hg background sample gave much higher recovery. Recovery in percentage was 25% at the analyzer, meaning a corrected recovery (adjusted for reduction of sample sizes during pipetting of the layers-this correction is called the use of a "pipette factor") of 30%, which is very low.

(6) Extraction Run #6

Control sediment samples were spiked with varying amounts of Me-Hg with extremely high Hg^{+2} background, to determine if any Hg^{+2} was getting through the extractions (not by nature of the solubility of the inorganic mercury, but by mistakes in pipetting). The samples, after extraction-oxidation procedure, registered nearly off scale. A redesign in pipetting procedures was made. Apparatus design was changed re the analysis vessel (for attachment to the MAS).

(7) Extraction Run #7

This was a reproduction of #6 with lower Hg^{+2} background (by factor of 10). More care was taken in pipetting; syringes were used for addition of some of the reagents. Linear reproducible recovery was obtained with high blanks. It was

here anticipated that background spiking of recoveries (with a knowledge of nature and approximate level of pollutant Hg in sample) would be necessary for meaningful analysis. Percentage recoveries were 41.5, 61.4, and 65.1 for 0.1, 0.3, and 0.5 µg Me-Hg addition, respectively. These are somewhat variable, but getting better. It seems that recoveries should be spiked with approximate level of Me-Hg in sample, if known, for more accurate analysis.

(8) Analysis Run #1

Extraction-oxidation-analysis was performed on the 25°C and the 54°C test-tube, one-gram, 100 ppm Hg^{+2} runs set up 23 days before. Because of a problem with sample size increasing throughout the oxidation (by addition of necessary reagents), aliquots were analyzed. Only two of the five 54°C samples gave any Me-Hg (742 and 90 ng/g). Only two of the five 25°C samples gave Me-Hg (213 and 91 mg/g). It was thus apparent that larger amounts of sediment would have to be used in modeling. There is really doubt about the data because the blanks and recoveries were also analyzed by aliquot. Consequently, the apparatus was redesigned to avoid similar problems in the future; an accurate calibration curve using it was prepared for the next run. Oxidizing of the cysteine layer was done at reduced room temperatures in all runs from here to avoid loss of mercury by volatilization.

(9) Extraction Run #8

In preparation of analysis of the 7-week 150 ppm Aquarium (I,II,III) runs, water and sediment Me-Hg- spiked samples were run with 50 ppm Hg^{+2} background. Linear recovery curves were obtained with 45% corrected recovery from the sediment and 90% corrected recovery from the water. Blanks gave a background of about 50 ng (again because of reagent contamination). The low recoveries were probably due to the use of a mechnical shaker rigged for the extractions rather than shaking by hand. Despite the low recoveries, they were linear and the machine reduced time of analysis from eight hours for 10 samples to five hours.

(10) Extraction Run #9

In an attempt to increase recovery a larger amount of concentrated HCl was added to samples of this run; this would increase chlorination of the Me-Hg, breaking its bonds with the thiol groups present in the sediment and thus increase the amount partitioned in the benzene layer of the extraction. Control sediment at 0.0 and 0.5 µg Me-Hg spikes gave lower corrected recovery of 34.1% with 53 mg blank value. The low recoveries (again in 50 ppm Hg^{+2} pollutant background) was thought to be caused by ineffective shaking.

(11) Analysis Run #2

Two recoveries were ran with three sediment/water samples of Aquarium II (Hg^{+2} pollutant 49-day run at room temperature, no aeration). No blanks were run as several previous runs

average about 53 ng. Corrected recoveries gave 64.3%. Back calculations on the Aquarium II samples gave 368, 310, and 346 mg/g. The average production in Aquarium II was thus 341 mg Me-Hg/g sediment.

(12) Analysis Run #3

Recoveries and samples from Aquariums I, II, and III were run through extraction-MAS analysis as above with blanks at the normal level. Recovery was 85.8%, possibly increased by the use of 1.5% cysteine HCl solution rather than the normal 1.0%. The results of the analysis in production of Me-Hg were:

Sample #1	Aquarium I	724 ng/g
2	Aquarium I	588 ng/g
3	Aquarium II	38.5 ng/g
4	Aquarium II	248 ng/g
5	Aquarium III	180 ng/g
6	Aquarium III	160 ng/g
7	Aquarium III	110 ng/g

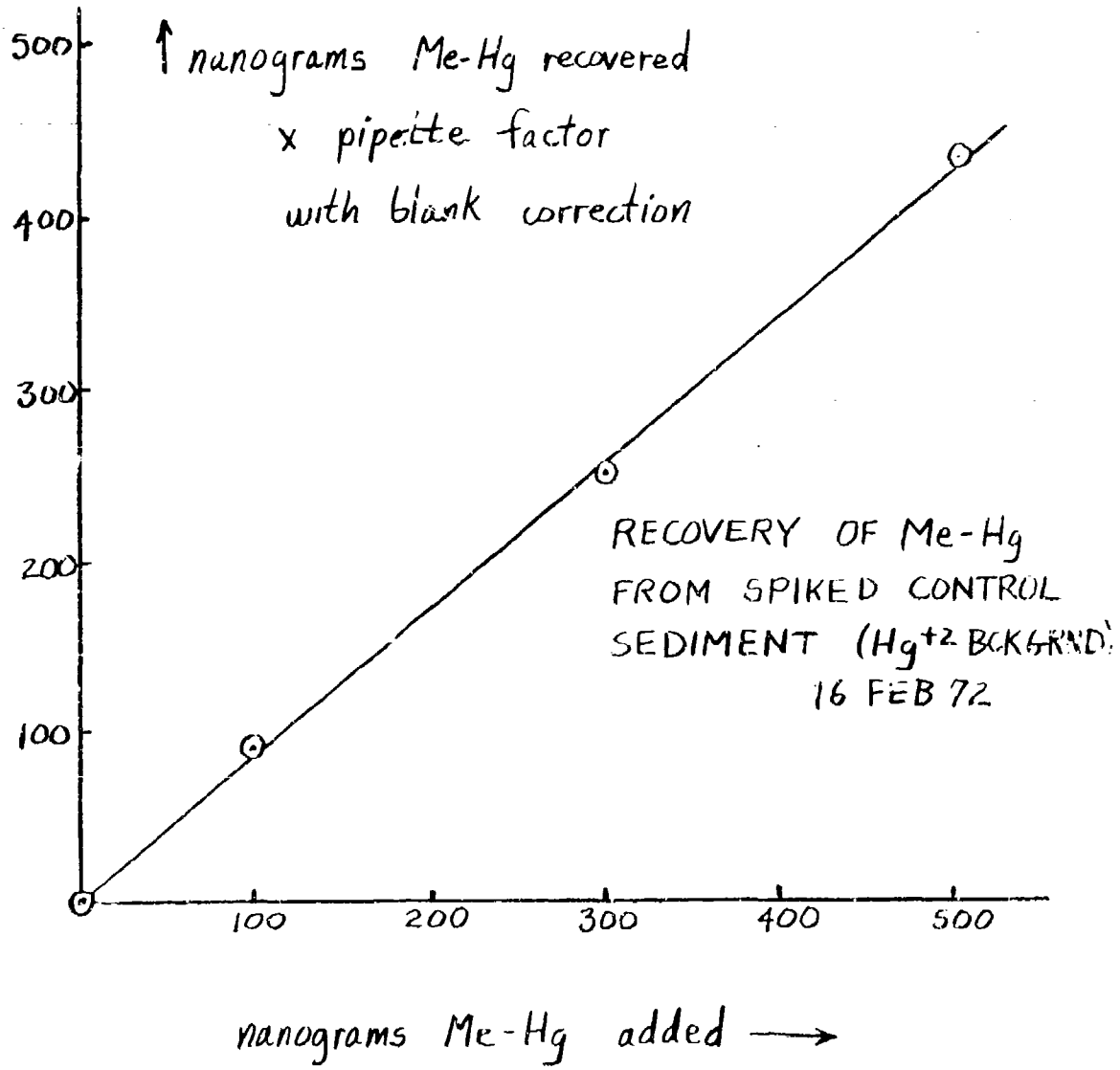
The samples in Aquarium II were taken through the decaying body of a larvae, averaging 143 ng/g. Aquarium I averaged 656 mg/g; Aquarium III averaged 150 mg/g.

(13) Extraction Run #10 - Analysis Run #4

These runs were attempts to verify linear recoveries by the method and to check the previous analyses of Aquarium I, II, and III. It was unsuccessful because the large number of samples, 22 not counting calibration standards, prolonged the analysis until an unknown factor of contamination ruined any possibility of obtaining meaningful results.

(14) Extraction Run #11

It was now desirable to confirm linear recoveries from various pollutant backgrounds. This run confirmed linear recovery of Me-Hg from 100 ppm Hg^{+2} background. The graph depicts the excellent results.



(15) Extraction Run #12

A linear recovery curve for phenyl-Hg^+ background was attempted and not obtained. The reason for this is unknown, but it could be that the solubility of the aryl ring in benzene might overcome the aqueous solubility of the inorganic end of the molecule, thereby allowing some of the pollutant to be pulled through to partitioning in the benzene layer of the first extraction. This would become significant at high phenyl-Hg^+ levels, supporting a need for spiking recoveries with approximate levels of pollutant (if known) as well as Me-Hg. The longer gas chromatography method, which is merely an extension to benzene partitioning of the acidified cysteine layer, may "filter out" this effect of pollutant level, but phenyl-Hg is analyzed by GC. If the samples from this run are averaged within recovery spike levels, linearity is obtained, but with very high blanks. Contamination may have been a factor and it was felt that, although this run could give some doubt as to the method when applied to phenyl-Hg backgrounds, the runs on the aquariums polluted with phenyl-Hg were not invalidated. To avoid any possibility, inorganic Hg was used as the sole pollutant for all remaining runs.

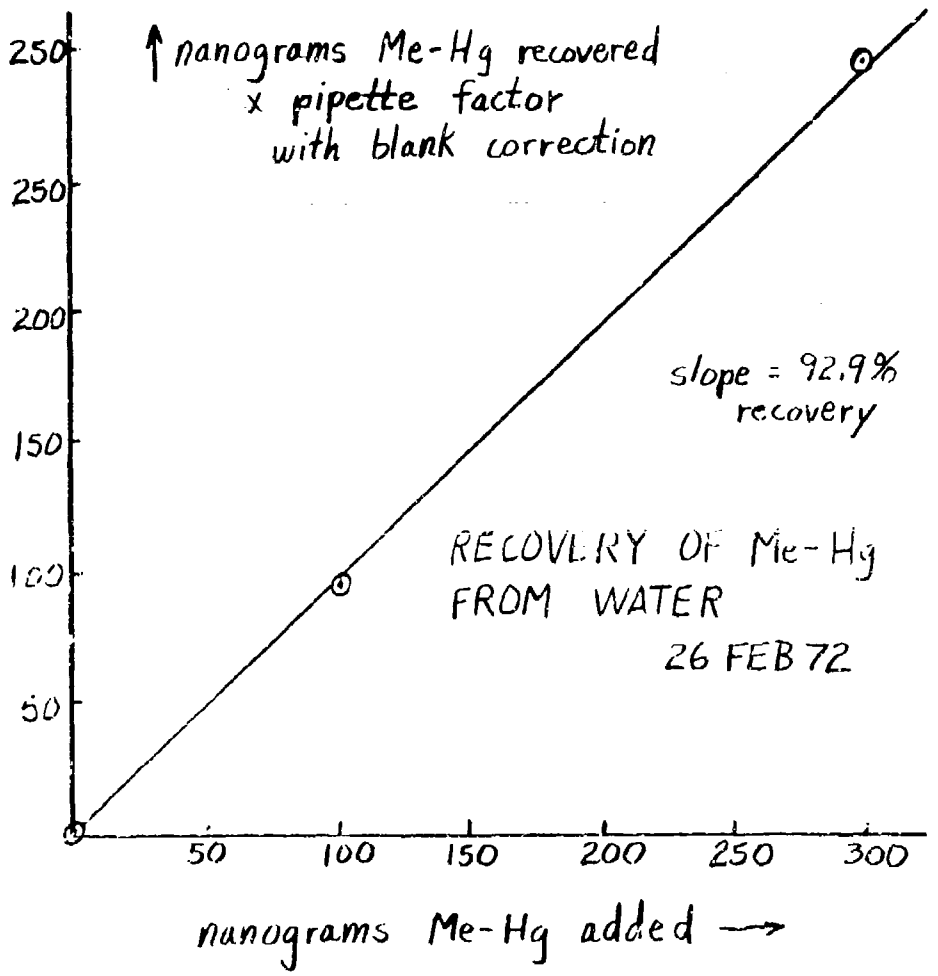
Also at this time the pH's of the three subject aquariums were measured and found to be for

Aquarium I pH = 5.1;
Aquarium II pH = 5.2;
Aquarium III pH = 5.5.

To try larger than one gram sediment samples for production of Me-Hg, small aquariums were set up with 30-40 g of Control sediment polluted to 100 ppm Hg^{+2}. These runs were later terminated because evaporation precipitated the $HgCl_2$ on the sides.

(1o) Extraction Run #13 - Analysis Run #5

In preparation for water analysis for Me-Hg in Aquarium II, a linear recovery was obtained from water samples:



Three samples of Aquarium II water were then run. Me-Hg content was lower than the blank recovery. The significance of this is indicated in the Discussion.

(17) Extraction Run #14

After stirring the water in Aquarium II, sediment samples, with no water taken from above it, were run through the extraction-analysis procedure for Me-Hg. No Me-Hg was found. The significance of this is indicated in the Discussion and the next work was orientated toward confirming a theory of Me-Hg binding that at this time seemed possible.

(18) Extraction Run #15

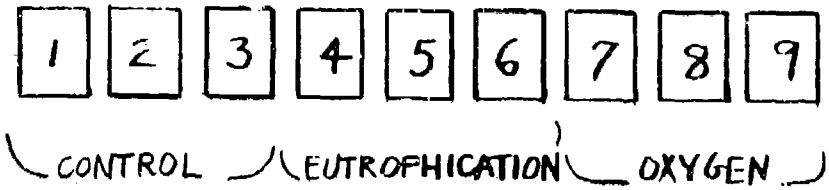
Water/sediment analysis for Me-Hg in Aquarium II was performed confirming its content is 320 mg/g.

(19) Analysis Run #6

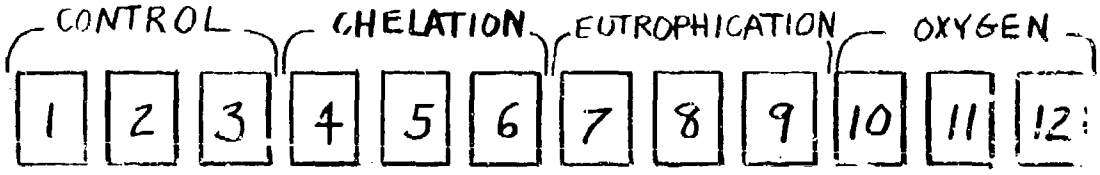
The 30-40 g aquariums were analyzed for Me-Hg at the 2-week point, with no indication of methylation. These runs were terminated as stated before.

In order to carry out the goal of investigating factors of methylation, now that methylation (in large aquariums) was confirmed, these runs were set up with approximately 5 ng of Rocky Gorge sediment:

MODERATE POLLUTION ~20 ppm Hg^{+2}



FACTORS AFFECTING CONVERSION



HEAVY POLLUTION ~200 ppm Hg^{+2}

The chelate NTA was added doubly-molar re the Hg^{+2} molar concentration. The eutrophication runs were aquariums spiked with artifical pollution (NO_3^- and PO_4^{-3}). Oxygen content was increased in the depicted aquariums by aeration. It was noted that visible life was killed by both levels of Hg^{+2} pollution, but began proliferating in three weeks.

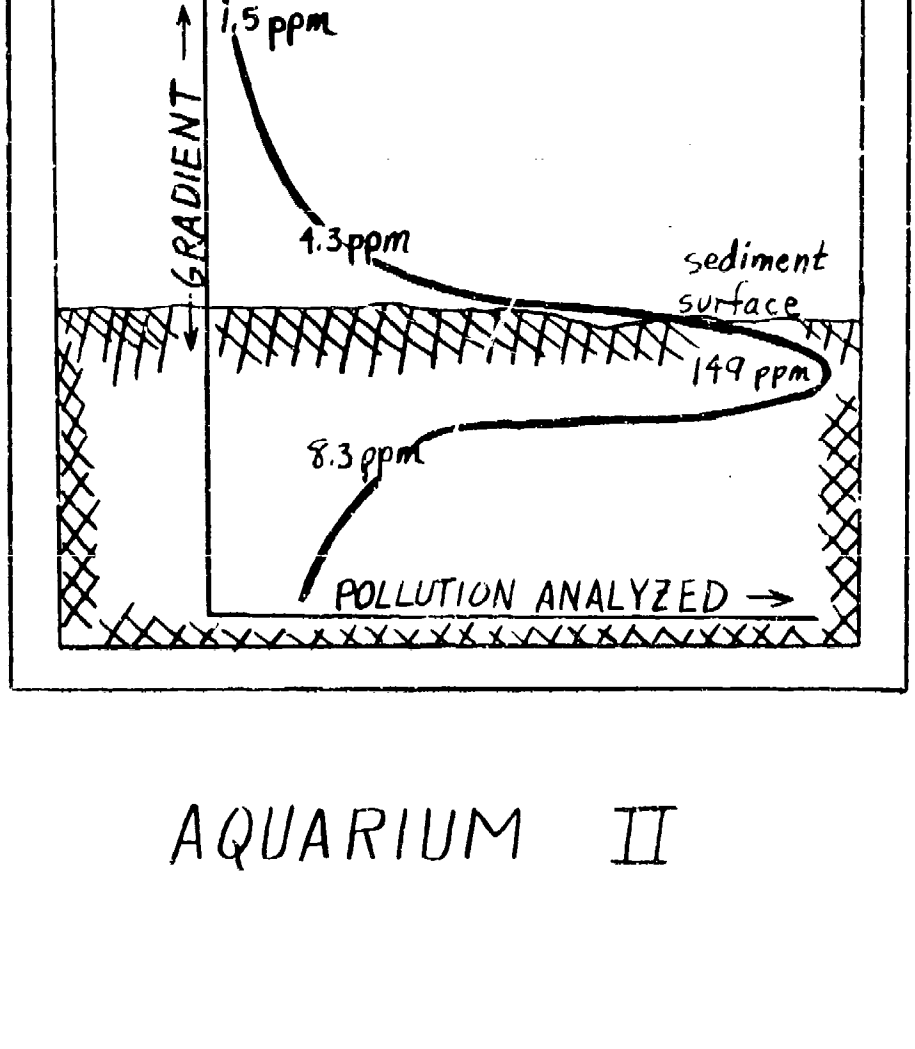
(20) Analysis Run #7 - Ultraviolet Spectrophotometric Detection of the Hg-NTA chelate.

After the method of Rollins[123], solutions of Hg^{+2} with and without the chloride anion, solutions of NTA, and a solution equimolar in Hg^{+2} and NTA were made up. On a Beckman DB-G UV spectrophotometer the solutions were run over the 400-200 mμ region, and the spectra compared. A shift in the absorption peaks by chelation could not be detected for two reasons: (1) the characteristic absorption peak at 2537Å (253.7 mμ) was so strong as to be off-scale for both Hg^{+2} and Hg-NTA solutions and the aliquoting/dilution method introduced uncertainties in actual concentration when attempts were made to lower the absorption peak. (2) the cuvettes began abosrbing at the 200 mμ end of the region.

This experiment did not disprove the formation of the Hg-NTA chelate; it just indicated that another method of detection should be employed, for which there was not time. It was found, as a side point, that the 2537Å peak was much attenuated (enough to be on scale) by formation of the chloride complex of Hg^{+2}. Attempts at confirming the Me-Hg cysteine binding (which is known) by the same method were unsuccessful for the same reasons given above.

(21) Analysis Run #8

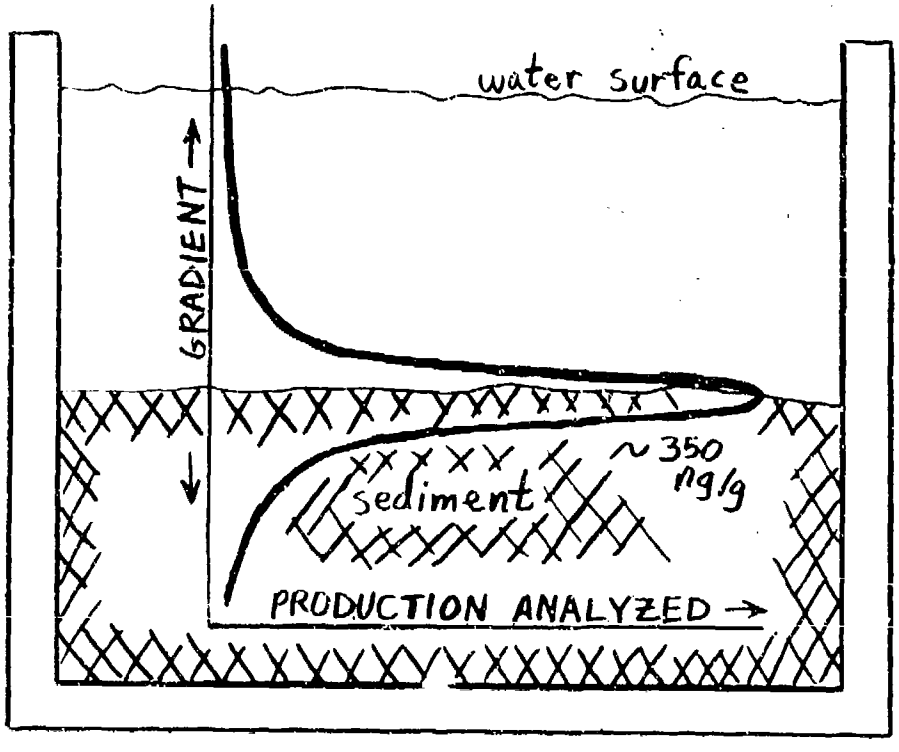
This was a total-Hg analysis of the gradient in Aquarium II, by the normal MAS-AA procedure of acid-oxidation of samples (see schematic at beginning of Experimental)



AQUARIUM II

(22) Analysis Run #9

This was a Me-Hg analysis of the gradient of production in Aquarium II by the extraction-analysis method. The results were partially indeterminant because of contamination but it was found that most of the Me-Hg was bound to the surface of the sediment. The significance of this is discussed in the summary.



AQUARIUM II

(23) Extraction Run #16

This was another investigation of pollutant-background effect on the Me-Hg analytical method. Varying concentrations of Hg^{+2} was placed in the Me-Hg recovery samples. Inconclusive results were obtained because of contamination by the H_2SO_4 stock acid (a new bottle had been opened).

Extraction by benzene of Aquarium II sediment was performed for Me-Hg detection and identification by the method of thin-layer chromatography . The attempt was unsuccessful because of lack of appropriate indicator reagent.

(24) Analysis Run #10

Samples of the new aquariums (set up four weeks before to investigate the factors of pollution level, chelation, entrophication, and oxygen content) were treated by the extraction-partitioning method for gas chromatography (reference has already been made) and taken to the laboratory of L. Kamps of the Pesticide Division of the Food and Drug Administration in Washington, D.C. No Me-Hg was detected because of failure to acidify the cysteine layer in the partitioning with the final benzene layer.

(25) Analysis Run #11

A duplicate of #10, the results of this analysis showed that recoveries averaged 71% but any Me-Hg production in the aquariums was masked by interferences from the drying agent

used in the last step. Me-Hg production would have had to have been more than 50 ng/g before the peak could have been seen.

(26) Analysis Run #12

In this the final analysis, an attempt at confirming the 341 ng/g Me-Hg production of Aquarium II sediment was made by the gas chromatography method.

Interferences were still present but Me-Hg from Aquarium II was identified at a level of about 30-40 ng/g, a factor of 1/10 the level found by the MAS-AA procedure used throughout the project.

VI. DISCUSSION

The experimental details of the project have been presented with explanations behind each individual experiment incorporated into the presentation. A discussion of the significance of these results will now be made.

The primary goal of research experience has, of course, been obtained. The goals of the project itself were to set-up a research project to investigate some of the factors involved in the conversion of mercury pollutants to toxic methylmercury by micro-organisms in marine sediments.

Research resulted in the definition of the two primary problems, modeling and analysis, and a proposal in solving them in an effort to carry out the goal of the project.

A successful modeling technique was then developed, after restrictions in design were found desirable. A successful analytical technique, which had not been applied as such to biological samples, was developed and successfully used.

These two solutions were then combined in an investigation of the conversion factors of:

(1) pollutant type and level
(2) conversion time
(3) movement of water over the sediment (removal of Me-Hg produced)
(4) chelation
(5) eutrophication
(6) oxygen content of the water above the sediment

It was found that the combination was safely applicable, in
in factor (1), to Hg^{+2} pollution up to even the high levels found in polluted sediments.

It was found that, in factor (2), conversion was undetectable in less than two weeks but quantitative after six weeks, occuring less rapidly in phenyl-Hg^{+} polluted sediment. This conclusion is limited, of course, to the single aquarium setup in our laboratories with a single sediment type at a single pollutant level.

It was found that, re factor (3), Me-Hg produced by a polluted sediment was, in this case, loosely bound so that stirring the water in the aquarium resulted in release of Me-Hg complexes from the sediment and dispersion into the water above the sediment. This could make Me-Hg more easily available to fish. This conclusion is based on the results of experiments 16, 17, 18, 22, and 23. However, the Hg^{+2} is apparently more tightly bound (indicated during modeling experiments by fish dying even after flushing the aquariums three times with water).

The results of investigation of the factors (4), (5), and (6) were inconclusive for reasons given in the Experimental. Me-Hg was definitely identified, though, at a significant level over Control sediment as a result of mathematical error, sampling (a single sample was analyzed in experiment (26)), or in the procedure that was developed in our laboratories (MAS-AA, i.e. the extraction-analysis method depicted at the beginning of the Experiment and used throughout the project).

The literature research has shown (in sections I, II, an III) that long-term mercury pollution results primarily from the solw conversion of large Hg^{+2} deposits tc toxic methyl-mercury by methanogenic micro-organisms found largely in marine sediments. An investigation into the biochemical reactions has shown that many pathways of conversion are possible under varying environmental conditions. Once some of the conditions were known, the project was defined and carried through to experimental results, some of which are significant to other researchers in the field and some of which are significant only in the training of a potential graduate student and in the spreading of knowledge of the investigation to interested persons at the Academy. Possibly the most significant result regarding this last fact is that our laboratories now have a method of mercury analysis, with the required special reagents, that might have not been available as soon if the project had not been carried out. The collection of knowledge alone gleaned from the research has undoubtedly benefited the Academy and the investigator.

VII. SUMMARY

1. A survey of the literature concerning mercury pollution has been made.
2. Factors involved in mercury poisoning have been presented and discussed.
3. Discussion of the methods involved in analysis for mercury has been presented with consideration of some of the problmes.
4. A research plan which was later modified was presented.
5. The setup of models which permit monitoring the production of methylmercury by microorganisms present in sediments has been described.
6. The development of a simple and rapid analytical method for mercury with specifity for alkyl mercury compound has been carried out.
7. The effect of selected factors on the production of methylmercruy was studied. These factors are (1) concentration of pollutant; (2) time of conversion; (3) removal of loosely bound methylmercury by water notion; (4) presence of chelating agents, (5) eutrophication (6), and (6) oxygen-content.
8. A bibliography with 157 references has been complied.

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